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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/70 — A1

(11) International Publication Number: WO 98/50047

(43) International Publication Date: 12 November 1998 (12.11.98)

(21) International Application Number: PCT/US98/09031

(22) International Filing Date: 8 May 1998 (08.05.98)

(30) Priority Data:

60/046,030	9 May 1997 (09.05.97)	US
60/061,716	10 October 1997 (10.10.97)	US

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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(54) Title: METHODS AND COMPOSITIONS FOR REDUCING ISCHEMIC INJURY OF THE HEART BY ADMINISTERING ADENOSINE RECEPTOR AGONISTS AND ANTAGONISTS

(57) Abstract

Compositions and methods for reducing or preventing ischemic damage of the heart are disclosed. A preferred embodiment of the invention comprises the simultaneous administration of specific A3/A1 receptor agonists, to patients suffering from ischemic damage or at risk for the same. In yet another embodiment of the invention, a binary conjugate which acts as an agonist for the A3 receptor and an antagonist at the A2a receptor, is administered to reduce or prevent ischemic damage to the heart.

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METHODS AND COMPOSITIONS FOR REDUCING ISCHEMIC INJURY OF THE HEART BY ADMINISTERING ADENOSINE RECEPTOR AGONISTS AND ANTAGONISTS

Pursuant to 35 U.S.C. §202(c) it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Institutes of Health, Grant Number HL48225.

FIELD OF THE INVENTION

The present invention relates to therapeutic methods for protecting the heart from ischemic injury. More specifically, the methods of the invention involve administering agonists and antagonists or binary conjugates thereof which selectively activate or inhibit adenosine receptors simultaneously thereby enhancing the protective effects of preconditioning and rendering the myocardium more resistant to ischemia.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by numerals in parenthesis in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

Adenosine is released in large amounts during myocardial ischemia and can mediate potentially important protective functions in the cardiovascular system (1,4,5,7,9,14, 17,18,19,25). Previous studies have shown that adenosine receptor agonists can precondition the heart when given before the onset of ischemia (4,5,9,14,17,18) and can cause reduction in

infarct size or improvement in left ventricular function when given during reperfusion (1,19) or during both low-flow ischemia and reperfusion in isolated perfused heart (6,21,22). While activation of adenosine A1 and A3 receptors has been shown to mimic the cardio-protective effect of preconditioning (3,10,23,24), their roles in mediating the protective effect of adenosine administered during ischemia have not yet been fully elucidated. Further, the cardioprotective effect of exogenous adenosine infused during ischemia in the intact heart may be exerted at the level of coronary vasculature, circulating neutrophils, or cardiac myocytes.

Our previous studies have characterized a cardiac myocyte model of injury, which is induced by exposure of myocytes to prolonged hypoxia in glucose-free media (16,23). Use of this model has facilitated the identification of compounds that enhance the protective effects of preconditioning and also increase myocardial resistance to ischemia.

SUMMARY OF THE INVENTION

The present invention provides methods for preventing or reducing ischemic damage of the heart. In conducting research leading up to this invention, it was discovered that simultaneous activation of A3 and A1 receptors enhances the protective effects of preconditioning and increases myocardial resistance to ischemia. The concept underlying the present invention is the use of specific agonists which simultaneously activate these two adenosine receptors. Concomitant activation of the two receptors is believed to produce a synergistic effect enhancing the cardioprotective effects of preconditioning and increasing myocardial resistance to ischemia.

According to a preferred embodiment, the invention involves administration of specific A1/A3 agonists, such

as N^6-(2-trifluoromethyl)(carbamoyl)adenosine-5'uronamide or N^6-(3-iodophenyl)(carbamoyl) adenosine-5'uronamide during ischemic attacks, or at risk for ischemic damage. The agonists of the invention may be delivered prior to a surgical procedure. They may also be administered to a patient to prevent or reduce the severity of ischemic damage during surgery. Additionally, the A3/A1 agonists may be administered following surgical procedures to reduce the risk of post-surgical ischemic complications. Finally, the A3/A1 agonists may be administered to patients with angina or to patients during a myocardial infarction. The angina may be chronic and stable, unstable, or post-myocardial infarction angina.

In yet another embodiment of the invention, a series of water-soluble MRS compounds are contemplated to be within the scope of the present invention. These compounds selectively activate the A3 receptor. Because the compounds of the invention do not cross the blood-brain barrier, the deleterious effects associated with A3 receptor activation in the brain are avoided. The MRS compounds will be used in conjunction with the A1 agonists of the invention to prevent or reduce ischemic damage to the heart.

Another preferred embodiment of the invention comprises novel binary conjugates which bind two adenosine receptors simultaneously. Exemplary binary conjugates of the invention contain moieties that act as agonists at both of the A1 and A3 adenosine receptors, such as MRS 1543. A second exemplary conjugate, MRS 1528, acts simultaneously as an agonist at the A3 receptor and as an antagonist at the A2a receptor. Methods are disclosed herein for the administration of these binary conjugates to protect the heart against ischemic damage.

Methods of simultaneous administration of the A3 and A1 agonists or the binary A3 agonist/A2a antagonist

or the binary A3 agonist/A1 agonist of the invention include direct perfusion of the organ during surgery and intravenous administration. Additionally, the agonists and antagonists of the invention may be administered to patients in tablet form in an amount effective to prevent or reduce ischemic damage to the heart.

In yet a further aspect of the invention, recombinant myocytes are provided which may be used to advantage in assessing the activity of agents that may possess cardioprotective activity. Cardiac myocytes may be transfected with any of the adenosine receptor encoding cDNAs and used to screen for novel therapeutic agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are graphs showing the effects of adenosine A3 receptor antagonists on the 2-chloro-N^6-cyclopentyl-adenosine (CCPA) and 2-chloro-N^6-(3-iodobenzyl)adenosine- 5'-N-methyluronamide (Cl-IB-MECA)-induced cardioprotective effect. Cultured ventricular myocytes were prepared and the extent of hypoxia-induced myocyte injury determined as described hereinbelow. The A3 antagonist, 3-ethyl-5-benzyl-2-methyl-6-phenyl -4-phenylethynyl- 1,4-(1)-dihydropyridine-3,5-dicarboxylate (MRS 1191) was present at the indicated concentrations individually, with CCPA (10 nM), or with Cl-IB-MECA (10 nM) during the ninety-minute hypoxia. The percentage of myocytes killed, Figure 1A and the amount of CK released, Figure, 1B, were determined following the prolonged hypoxia. Data represent the mean ±SE of three experiments.

Figure 2 is a graph showing effects of adenosine A1 agonists, 2-chloro-N^6-cyclopentyladenosine (CCPA), N^6-cyclohexyladenosine (CHA), and adenosine amine congener (ADAC) on cardiac myocyte injury in the

presence or absence of excess 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an A1 antagonist. (open squares, CCPA; open triangles, CHA, open circles, ADAC; filled squares CCPA + DPCPX; filled triangles, CHA + DPCPX; filled circles, ADAC + DPCPX)

Figures 3A-3F are graphs illustrating the cardioprotective effects of the MRS compounds of the invention. Figure 3A shows the amount of creatine kinase released as a function of increasing concentrations of MRS 584 in the presence (diamonds) or absence (open squares) of the A1 receptor antagonist DPCPX. Figure 3B shows the amount of creatine kinase released as a function of increasing concentrations of MRS 537 in the presence (diamonds) or absence (open squares) of the A1 receptor antagonist DPCPX. Figure 3C shows the amount of creatine kinase released as a function of increasing concentrations of MRS 479 in the presence (diamonds) or absence (open squares) of the A1 receptor antagonist DPCPX. Figure 3D shows the amount of creatine kinase released as a function of increasing concentrations of MRS 1340 in the presence (diamonds) or absence (open squares) of the A1 receptor antagonist DPCPX. Figure 3E shows that both MRS584 and MRS537 can exert the cardioprotective effect of preconditioning via the human adenosine A3 receptor. Data represent means ± SE of three experiments. Figure 3F shows that Cl-IB-MECA reduces the percentage of myocytes killed during ischemia.

Figures 4A, and 4B are graphs showing the reduction in cardiac myocyte cell death following simultaneous exposure to A1 and A3 agonists on cardiac cells. Figure 4A shows that the preconditioning effect is synergistically enhanced when adenosine A1 and A3 receptors are activated simultaneously, as opposed to activation of a single receptor. The percentage of

myocytes killed is also significantly reduced in the simultaneous presence of A1 and A3 agonists during prolonged ischemia. See Figure 4B. Data represent means ± SE of four experiments.

Figures 5A- 5D are graphs showing the cardioprotective effects mediated by MRS 646 and MRS 1364 which activate the A1 and A3 receptors simultaneously. Figure 5A shows that MRS646 and MRS1364 can pharmacologically precondition the cardiac myocyte. The extent of myocyte injury was quantitated as percentage of myocytes killed. The data show that when either MRS646 or MRS1364 was present during the injury-producing ischemia, both compounds protect against injury. See Figure 5B. Figure 5C shows that MRS 1364 was able to pharmacologically precondition the cardiac myocytes transfected with the human adenosine A3 receptor. Data represent the means ± S.E. of three experiments. Figure 5D shows that MRS 646 was able to pharmacologically precondition the cardiac myocytes transfected with the human adenosine A3 receptor. Data represent the means ± S.E. of three experiments.

Figures 6A, 6B and 6C are graphs that show the cardioprotective effects of MRS1543, a binary A1/A3 agonist. Figure 6A shows that the protective effect of MRS1543 was only partially attenuated by DPCPX or by MRS1191. Ventricular myocytes were exposed to the indicated concentrations of MRS1543 in the presence or the absence of excess DPCPX (1 μM) or excess MRS1191 (1 μM). The ventricular myocytes were also exposed to the indicated concentrations of MRS1543 in the presence or the absence of excess DPCPX (1 μM) plus excess MRS1191 (1 μM). See Figure 6B. The combined presence of both DPCPX and MRS1191 completely abolished the protective effect of MRS1543. The protective effect of MRS1543 in chick cells transfected with the human adenosine A3

receptor is shown in Figure 6C. Data represent means ± SE of three experiments.

Figures 7A and 7B are graphs showing the cardioprotective effects of simultaneous administration of an A3/A1 agonist, MRS 580, and an A_{2a} antagonist on myocyte survival. Figure 7A shows that an A_{2a} antagonist enhances the ability of an A3/A1 agonist, MRS 580, to cause preconditioning. Figure 7B shows that an A_{2a} antagonist enhances the protective effect of MRS 580 during prolonged ischemia. Figure 7C shows that MRS 580 can also precondition the cardiac myocyte via the human adenosine A3 receptor.

Figures 8A-8E are a series of graphs showing the cardioprotective effects of MRS1528, a binary conjugate having agonist activity at the A3 receptor and antagonist activity at the A2a receptors. Figure 8A shows that the ability of MRS1528 to cause preconditioning is not affected by the presence of 8-(3-chlorostyryl)caffeine, (CSC). Myocytes were exposed to the indicated concentrations of MRS1528 in the presence or the absence of CSC (1 μM). Myocytes were also exposed to the indicated concentrations of MRS1528 in the presence or the absence of CGS21680 (0.3 μM). See Figure 8B. Figure 8C shows the results obtained when myocytes were exposed to MRS1525 in the presence or the absence of CSC. Figure 8D shows the results obtained when myocytes were exposed to the indicated concentrations of MRS1525 in the presence or the absence of CGS21680. The preconditioning effect of MRS1528 in cells transfected with the human adenosine A3 receptor is shown in Figure 8E. Data represent the means ± SE of three experiments.

Figure 9 is a diagram showing the synthetic scheme utilized to generate the binary compounds used in the

practice of this invention.

Figures 10A-10C depict diagrams for synthesizing certain compounds of the invention. Figures 10A and 10B show a synthetic scheme for synthesizing a derivative of an A1 selective agonist for coupling to an amine derived A3 agonist. Figure 10C is a schematic diagram showing the synthesis of binary conjugates with extended linkers.

Figures 11A and 11B show the nucleotide sequence of the cDNA encoding the adenosine A1 receptor.

Figures 12A-12D show the nucleotide sequence of the cDNA encoding the adenosine A2a receptor.

Figures 13A-13C show the nucleotide sequence of the cDNA encoding the adenosine A3 receptor.

DETAILED DESCRIPTION OF THE INVENTION

The cardioprotective roles of adenosine A1 and A3 receptors were investigated in a cardiac myocyte model of injury. The adenosine A3 receptor is a novel cardiac receptor capable of mediating potentially important cardioprotective functions. Prolonged hypoxia with glucose deprivation was used to simulate ischemia and to induce injury in cardiac ventricular myocytes cultured from chick embryos 14 days in ovo. When present during the prolonged hypoxia, the adenosine A3 agonists N6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) and Cl-IB-MECA caused a dose-dependent reduction in the extent of hypoxia-induced injury, as manifested by a decrease in the amount of creatine kinase released and the percentage of myocytes killed. The adenosine A1 agonists CCPA and N6-cyclohexyladenosine (CHA) were also able to cause a decrease in the extent of myocyte injury. The A1

receptor-selective antagonist DPCPX blocked the cardioprotective effect of the A1 but not of the A3 agonists. Conversely, selective A3 antagonists MRS1191 and 3,5-Diethyl 2-methyl-6-phenyl-4-[2-phenyl-(E)-vinyl]-1,4-(1)-dihydropyridine-3,5-dicarboxylate (MRS 1097) blocked the protection induced by Cl-IB-MECA but had minimal effect on that caused by CCPA. Thus, the cardioprotective effects of A1 and A3 agonists were mediated by their respective receptors. The study identifies the cardioprotective function of the cardiac A3 receptor and provides conclusive evidence that simultaneous activation of both A1 and A3 receptors during hypoxia can attenuate myocyte injury. This finding is in contrast to that set forth by Downey et al. in U.S. Patent No. 5,573,772 wherein administration of antagonists to the A1 receptor in conjuction with agonists at the A3 receptor was reported to enhance cardioprotective effects during ischemia. Administration of an adenosine A1 receptor antagonist is not required for practicing the present invention.

The present invention also includes administration of binary reagents that selectively activate the A1 and A3 adenosine receptors simultaneously. Concomitant activation of the two receptors is believed to act synergistically to enhance the cardioprotective effects of preconditioning and to increase myocardial resistance to ischemia.

Several new MRS compounds are disclosed which selectively activate the A3 adenosine receptor. See Figures 3A-3F. These compounds are also contemplated for use in the methods of the present invention.

In accordance with another aspect of the invention, a binary conjugate has been developed, which binds both the A3 and A2a receptors simultaneously and elicits the desired result, i.e., activation of the A3 receptor and inhibition of the A2a receptor.

A second binary conjugate, has also been

synthesized in accordance with this invention which simultaneously binds and activates both of the A1 and the A3 receptors. These binary compounds may also be used to advantage in practicing the methods of the present invention. The following definitions are provided to facilitate understanding of the present invention.

Preconditioning ischemia - A brief ischemia which does not cause any cardiac damage, but is able to protect the heart against damage during a subsequent prolonged ischemia. The effect of preconditioning ischemia is mediated by adenosine, which is released during the ischemia. Preconditioning may be induced by brief exposure to anoxic conditions for example.

Adenosine receptors - A1, A3 and A2a receptors are present on the myocardium (cardiac muscle cells). While activation of the A1 and A3 receptors is cardioprotective, activation of the A2a receptors is deleterious and causes damage to the cardiac muscle cells.

Stable angina - Condition observed in certain cardiac patients having a chronic risk for mycardial ischemia because of the chronic potential for an imbalance between the supply of oxygenated blood and the demand for it. Typically, such imbalance occurs during certain stresses, such as exercise, emotional stress or stress associated with a surgical procedure.

Unstable angina - Condition observed in cardiac patients having frequent imbalance between the supply of and the demand for oxygenated blood.

Post-myocardial infarction angina - Condition observed in patients who have recurrent ischemia following a

heart attack.

Preconditioning stimuli - Any drug, agent or treatment which induces preconditioning, such as brief ischemia, or A1 or A3 receptor agonists.

Myocardial responsiveness - The myocardium can be treated so as to enhance the effectiveness and protective effects of preconditioning. This enhancement leads to a reduction in ischemic damage.

Representative examples of compounds used for practicing the present invention are as follows
A1 agonists - CCPA, and compounds listed in Table I.
A1 antagonists - DPCPX
A3 agonists - IB-MECA, Cl-IB-MECA, MRS 584, MRS 537, MRS 479, MRS 1340, DBXMR, NNC21-0238, NN53-0055
A3 antagonists - MRS 1191
A2a agonists - CGS21680
A2a antagonists - CSC
Binary A3/A1 agonists - MRS 1543
Binary A3 agonist/A2a antagonists - MRS 1528
Mixed A3/A1 agonists - compounds listed in Table II, including MRS 580 and MRS 1364

The present invention demonstrates that activation of adenosine receptors during prolonged hypoxia can attenuate the hypoxia-induced myocyte injury. Specifically, activation of A1 and A3 receptors has been shown to mediate adenosine-induced cardiac myocyte protection. Additionally, the concomittant activation of the A3 receptor coupled with inhibition of A2a receptor activation by a selective binary conjugate has also been shown to attenuate hypoxia-induced myocyte injury. The following methods facilitate the practice of the present invention.

Preparation of Cultured Ventricular Cells

Ventricular cells were cultured from chick embryos 14 days in ovo as previously described (16, 23). Cells were cultivated in a humidified 5% CO_2-95% air mixture at 37°C. All experiments were performed on day 3 in culture, at which time cells exhibited rhythmic spontaneous contraction. The medium was changed to a HEPES-buffered medium containing (mM) 139 NaCl, 4.7 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 5 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 2% fetal bovine serum, pH 7.4, 37°C before exposing the myocytes to the various conditions at 37°C. Control myocytes were maintained in the HEPES-buffered media under room air. Ninety-minute exposure of the myocytes to hypoxia with glucose deprivation was used to induce cell injury. Hypoxia was produced by placing the cells in a hypoxic incubator (NuAire) where O_2 was replaced by N_2 as previously described (16,23). Effects of adenosine receptor agonists and antagonists on the extent of myocyte injury were determined by exposure of the prepared cells to these agents during the prolonged hypoxia.

Determination of Cell Injury

Determination of myocyte injury was made at the end of the ninety-minute hypoxia, at which time myocytes were taken out of the hypoxic incubator and re-exposed to room air (normal % O_2). Aliquots of the media were then obtained for creatine kinase (CK) activity measurement, which is followed by quantitation of the number of viable cells. Measurement of basal level of cell injury was made after parallel incubation of control cells under normal % O_2. The extent of hypoxia-induced injury to the ventricular cell was quantitatively determined by the percentages of cells killed and by the amount of CK released into the media according to a previously described method (16, 23). Prior studies demonstrated that the cell viability assay

distinguished the hypoxia-damaged from the control normoxia-exposed cells. In brief, the media were replaced with a trypsin-EDTA buffer to detach the cells, which was then followed by sedimentation of the viable myocytes. Parallel changes in % cells killed and CK released (16, 23) further validated this assay for % cells killed. The amount of CK was measured as enzyme activity (unit/mg), and increases in CK activity above the control level were determined. The percentage of cells killed was calculated as the number of cells obtained from the control group (representing cells not subjected to any hypoxia or drug treatment) minus the number of cells from the treatment group divided by number of cells in control group multiplied by 100%.

Synthesis of Adenosine A1 and A3 Receptor-selective Agents N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (Cl-IB-MECA), and adenosine amine congener (ADAC) were synthesized as previously described (8,11,13). 3,5-Diethyl 2-methyl-6-phenyl-4-[2-phenyl-(E)-vinyl]-1,4-(1)-dihydropyridine-3,5-dicarboxylate (MRS 1097) and 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(1)-dihydropyridine-3,5-dicarboxylate (MRS 1191) were synthesized as previously described (12).

Adenosine analogs

8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 2-chloro-N^6-cyclopentyladenosine (CCPA), N^6-cyclohexyladenosine (CHA) were from Research Biochemicals International (Natick, MA). Embryonic chick eggs were from Spafas Inc. (Storrs, Conn).

Transfection of chick atrial myocytes with the human A3 receptor - Atrial cells were cultured from chick embryos 14 days in ovo and transiently transfected with pcDNA3 (empty vector) or with pcDNA3/hA3R (full length cDNA encoding human adenosine A3 receptor subcloned in pcDNA3) using the calcium phosphate precipitation method. Forty-eight hours after transfection, the cells were exposed to the compounds of the invention and the percentage of myocytes killed during simulated ischemia was determined.

The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

EXAMPLE I
ACTIVATION OF A1 OR A3 ADENOSINE RECEPTORS REDUCES ISCHEMIC INJURY TO THE HEART

A. Exogenous adenosine causes a decrease in the extent of cardiac myocyte injury during the prolonged hypoxia

Prolonged exposure to hypoxia in glucose-free media induced significant cardiac myocyte injury with large increase in the release of creatine kinase (28.5 ± 1.5 unit/mg, n= 4, ± SE) and in the % cells killed (30 ± 2 %, n= 4). Adenosine (10 μM), when added to the media during the ninety-minute hypoxia, caused a decrease in the amount of CK released (CK released in unit/mg = 14.9 ± 3, n= 3) and in the % cells killed (% cells killed= 12 ± 2 %, n= 4). This effect of exogenous adenosine was blocked by the nonselective adenosine receptor antagonist 8-sulfophenyl-theophylline (8-SPT, at 100 μM) (in the presence of adenosine and 8-SPT: CK released in unit/mg = 31 ± 5 and % cells killed = 28 ± 3 %, n=5). The presence of 8-SPT during the hypoxia had no effect on the level of myocyte injury (CK released= 29.6 ± 1.2 unit/mg; % cells killed= 31.6 ± 1.5 %, n=3). These data

suggest that activation of adenosine receptors during the prolonged hypoxia can protect the myocyte against injury. Since adenosine can activate both the cardiac A1 and A3 receptors and since 8-SPT, at 100 μM, can block both receptors (23), the data are consistent with the hypothesis that either receptor or both receptor subtypes can mediate the cardioprotective function of adenosine. This hypothesis was further explored using selective agonists and antagonists. See Figures 1, 2 and 3.

B. Adenosine A3 agonists mediate cardioprotective effects during prolonged hypoxia

In order to examine whether activation of adenosine A3 receptors is capable of attenuating myocyte injury during the prolonged hypoxia, agonists selective at the A3 receptor were used. Prior study has demonstrated that both IB-MECA and Cl-IB-MECA are highly selective at the chick cardiac A3 receptor (23). Either A3 agonist, when present during the prolonged hypoxia, was capable of protecting the cardiac myocytes against hypoxia-induced injury (Figure 1). The cardioprotective effect of A3 agonists was quantitated as a decrease in the amount of CK released and the percentage of myocytes killed (statistically significant at 1 and 10 nM of Cl-IB-MECA, ANOVA and t test, $p<0.01$).

The presence of the A1 receptor-selective antagonist DPCPX had no effect on the ability of IB-MECA or Cl-IB-MECA to mediate their cardioprotective effects; the A3 agonist-induced decreases in % cells killed and CK released were similar in the presence and absence of 1 μM of DPCPX (not shown). These data indicate that the cardioprotective effect of the A3 agonists was not due to activation of the cardiac A1 receptor.

To determine whether the A3 agonist-induced cardioprotection is mediated by the A3 receptor, antagonists selective at the A3 receptor were employed.

Figure 1 demonstrates that the A3 receptor-selective antagonist MRS 1191 blocked the Cl-IB-MECA-induced cardioprotection. The levels of CK released and of percentage of cells killed were significantly higher in myocytes exposed to Cl-IB-MECA and 30 nM, 300 nM or 3 μM of MRS 1191 (ANOVA and t test, $p<0.01$). Another A3 receptor antagonist, MRS1097, was also able to block the cardioprotective effect of Cl-IB-MECA (not shown). These data provide conclusive evidence that activation of the A3 receptor can produce a potent cardioprotective effect when administered during the prolonged hypoxia.

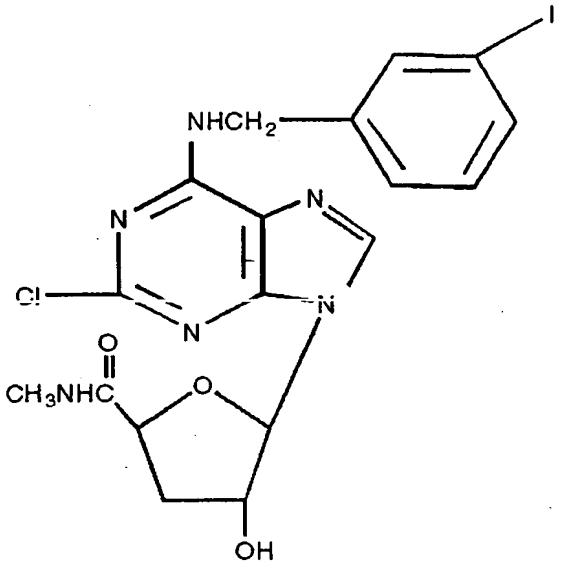
C. Adenosine A1 receptor activation reduces cardiac myocyte injury during prolonged ischemia

Since the A1 receptor is also present on the cardiac myocyte, an investigation was undertaken to determine whether activation of the A1 receptor can confer a cardioprotective effect during the prolonged hypoxia. Prior study showed that CCPA, a known A1 agonist, is highly selective at the A1 receptor on these cardiac myocytes (23). Cultured ventricular myocytes were prepared and the extent of hypoxia-mediated myocyte injury determined. The various adenosine A1 receptor agonists were added to the media at the indicated concentrations in the absence or the presence of the A1 receptor antagonist DPCPX during the prolonged hypoxia. The percentage of cells killed was determined following the hypoxic exposure and removal of the A1 receptor agonists and antagonist. The data represent the mean of four experiments. At 1 and 10 nM of the A1 agonists, the percentages of myocytes killed were significantly lower than those obtained in the presence of either of the two A1 agonist concentrations and DPCPX (1 μM) (ANOVA and t test, $P<0.01$). Thus, CCPA caused a dose-dependent reduction in the percentage of myocytes killed as shown in Figure 2 and in the amount of CK released (not shown) (ANOVA and t test, $p<0.01$). Two

other A1 receptor-selective agonists, ADAC and CHA, were also able to protect the myocytes when present during the prolonged hypoxia. The cardioprotection stimulated by CCPA, CHA and ADAC was blocked by the A1 antagonist DPCPX. On the other hand, neither MRS 1191 nor MRS 1097 was able to block the CCPA-induced cardioprotection as shown in Figure 1, providing definitive evidence that the A1 agonist effect is mediated by the A1 receptor.

Although the number of viable cells was determined quickly following re-exposure of cardiac myocytes to normal % O_2 (reoxygenation), the A1 or the A3 agonist was nevertheless present briefly prior to replacement with the trypsin-EDTA buffer for cell viability assay. Thus, it is possible that the decrease in myocyte injury is due to the protection against a reoxygenation injury. To study this possibility, CCPA or Cl-IB-MECA was added immediately upon reoxygenation following the ninety-minute hypoxic exposure. CCPA or Cl-IB-MECA was maintained in the media for an additional hour prior to determination of the percentage of myocytes killed. Although CCPA or Cl-IB-MECA was able to protect the myocytes when present during the reoxygenation, the extent of protection was small (% myocytes killed following the 90 minute hypoxia= 26.5 ± 1.0 %, n=6, ± S.E. vs. CCPA present= 22.1 ± 1.5 %, n=5 or vs. Cl-IB-MECA present= 23.0 ± 1.4 %, n=5; ANOVA and t test, $P<0.05$) The previous example illustrates the efficacy of A3/A1 adenosine receptor agonists in reducing ischemic damage to the heart. A variety of other compounds have been developed which also stimulate

A3 adenosine receptors. These are set forth below:



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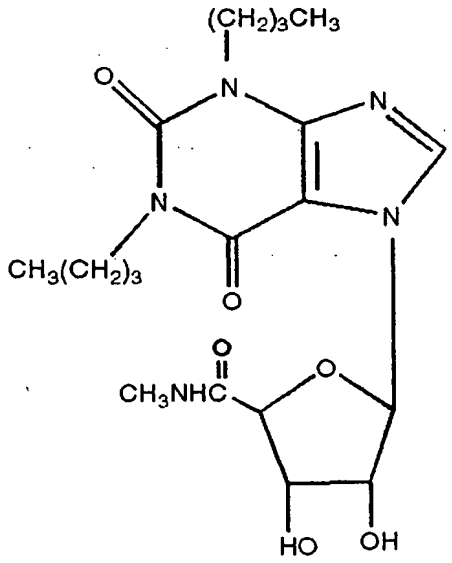
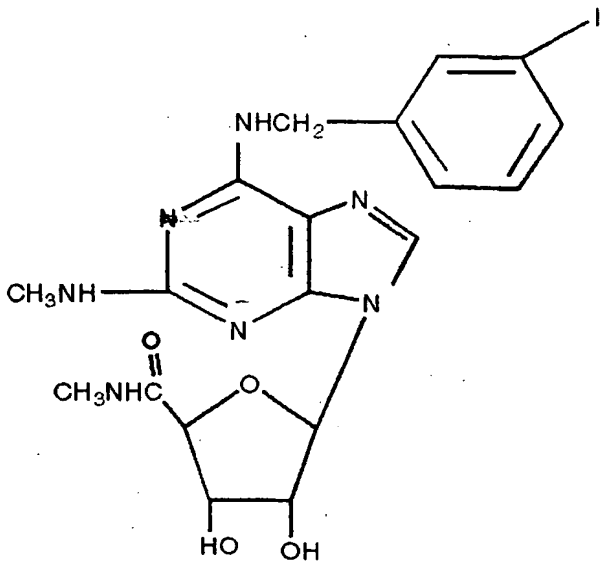
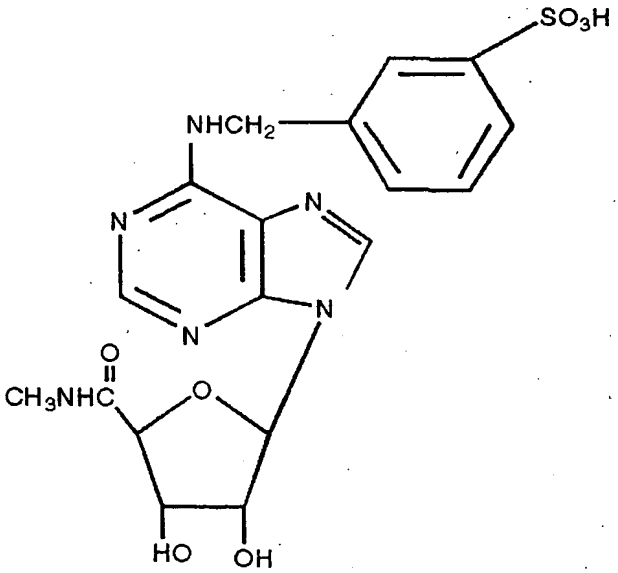


MRS 479

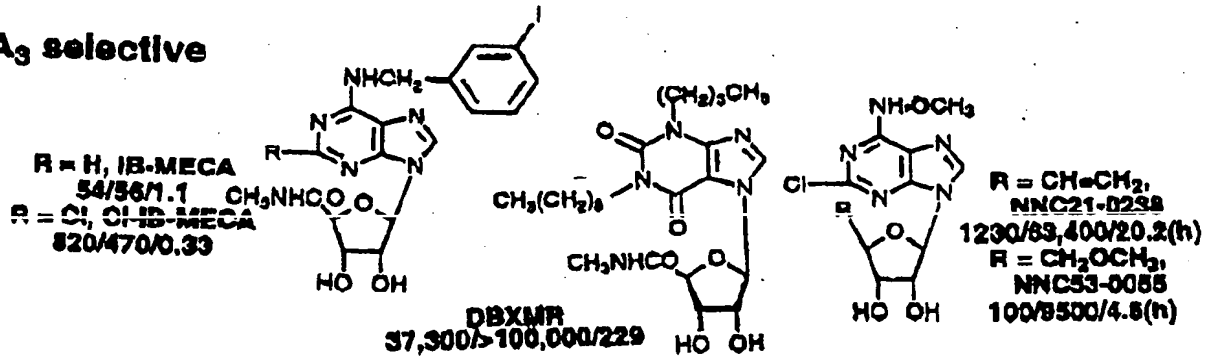


MRS 537



MRS 1340

Additional A3 selective agonists are set forth below:



The compounds shown immediately above have been assayed for cardioprotective efficacy during prolonged ischemia in the in vitro culture system described in Example I.

The cardioprotective effects of A3 receptor agonists MRS 584, MRS 537, MRS 479, MRS 1340 were assessed in the presence or absence of the A1 receptor antagonist DPCPX. The A1 receptor antagonist was utilized to demonostrate the selectivity of the MRS compounds for the A3 as opposed to the A1 adenosine receptor. Figure 3A shows the cardioprotective effects of MRS 584 in the presence and absence of DPCPX. The extent of myocyte injury was plotted as the amount of creatine kinase released during the prolonged simulated ischemia. Figure 3B shows the cardioprotective effects of MRS 537 in the presence and absence of DPCPX. The

extent of myocyte injury was plotted as the amount of creatine kinase released during the prolonged simulated ischemia as a function of increasing the concentrations of MRS 537. Figure 3C shows the cardioprotective effects of MRS 479 in the presence and absence of DPCPX. The extent of myocyte injury was plotted as the amount of creatine kinase released during the prolonged simulated ischemia as a function of increasing the concentrations of MRS 479. Figure 3D shows the cardioprotective effects of MRS1340 in the presence and absence of DPCPX. The extent of myocyte injury was plotted as the amount of creatine kinase released during the prolonged simulated ischemia, as a function of increasing the concentrations of MRS1340.

To determine whether the protective effects of the compounds of the invention would also comparably stimulate the human adenosine A3 receptor, atrial cardiac myocytes, which express little if any endogenous A3 receptor, were transfected with either empty vector or vector containing the human adenosine A3 receptor cDNA. The transfected myocytes were then exposed to the compound for 5 minutes, which was followed by replacement with fresh media and myocytes exposed to normal conditions (non-ischemic) for 10 minutes prior to being exposed to ninety minutes of ischemia. The decrease in the number of myocytes killed in A3 receptor cDNA-transfected myocytes as compared to vector-transfected myocytes was plotted as a function of the concentration of the compound. This decrease in myocytes killed represented an index of the protection mediated via the human adenosine A3 receptor.

Figure 3E shows the protective effects of MRS 584 and MRS 537 obtained when chick cells were transfected with a human A3 receptor encoded cDNA. These data show that the MRS compounds of the invention specifically bind to and activate the human A3 receptor.

Figure 3F shows that chick atrial cells aquire A3-

receptor mediated cardioprotective responses upon transfection with human A3 receptor encoding cDNA. Cells were exposed to 10 nM Cl-IB-MECA and 1 μM DPCPX for five minutes. Media were replaced with fresh media lacking Cl-IB-MECA or DPCPX. Cells were then incubated under room air for thirty minutes prior to being exposed to 90 minutes of simulated ischemia. The graphs shows the results obtained in cells transfected with A3 receptor encoding cDNA, untransfected cells and cells transfected with empty vector. In untransfected cells or cells transfected with empty vector, the A3 agonist was able to reduce the percentage of cells killed when compared to cells not pre-exposed to A3 agonist (control cells, * t-test $P<0.05$). However, the A3 receptor mediated reduction in the number of cells killed or the amount of CK released, expressed as % decrease from those obtained in the control cells, was significantly greater in the cells transfected with the human A3 receptor encoding cDNA than in untransfected or cells transfected with empty vector (one-way ANOVA and t test $P<0.01$**)

These data illustrate that these compounds selectively activate human adenosine A3 receptor and can be used to prevent ischemic damage to the heart. Each of the above described MRS compounds is contemplated for use in combination with any of the A1 agonists described herein for reducing ischemic damage to the heart.

EXAMPLE II

ADENOSINE A1 AND A3 RECEPTOR AGONISTS ACT SYNERGISTICALLY TO INDUCE CARDIOPROTECTION.

Adenosine can exert two principal cardioprotective effects. Adenosine can precondition the heart with reduction in the size of myocardial infarction (4,5,9,14,17,18). Intracoronary administration of

adenosine during reperfusion following prolonged no-flow ischemia can also limit infarct size in the intact heart (1, 19). Our previous studies have characterized a cardiac myocyte model of injury, which is induced by exposure of myocytes to a prolonged period of hypoxia in glucose-free media (16, 23). Activation of either the A1 or the A3 receptor by their respective agonists can pharmacologically precondition the cardiac myocyte (23). Figures 1 and 2 show that the presence of A1 or A3 agonist can also protect the cardiac myocytes when the agonist is present individually during an actual injury-inducing ischemia.

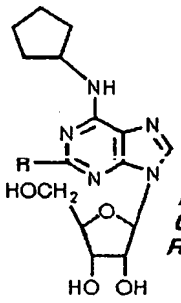
Figure 4A shows that the simultaneous activation of both A1 and A3 receptors produces a greater preconditioning effect than when either receptor is activated separately. Thus, an A1 agonist and an A3 agonist interact synergistically to precondition the cardiac myocyte. In addition, the simultaneous presence of A1 and A3 receptor agonists during injury-producing ischemia resulted in less myocyte injury than when only A1 or A3 agonist is present. See Figure 4B. Thus, simultaneous administration of A1 and A3 agonists will lead to enhanced cardioprotection.

Based on the data presented above, it is apparent that a variety of A1 agonists and A3 agonists may be used in conjunction to reduce or prevent ischemic damage to the heart. Suitable A1 agonists contemplated for use in the present invention are set forth below in Table I.

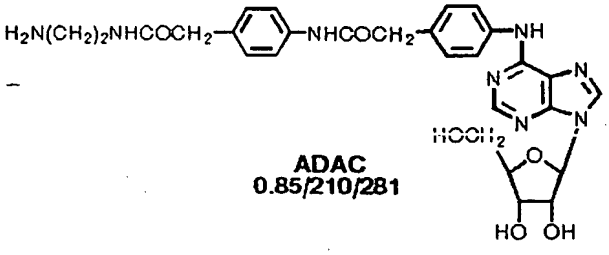
TABLE I

A1 AGONISTS

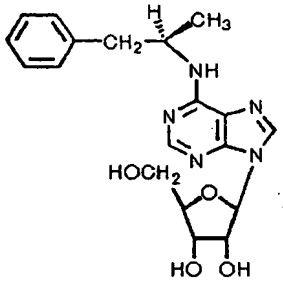
A_1 selective



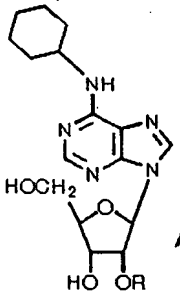
R = H, CPA
0.59/460/240
R = Cl, CCPA
0.6/950/237



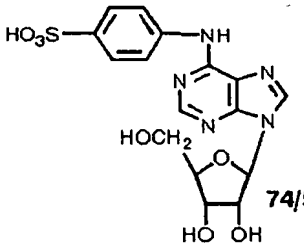
ADAC
0.85/210/281



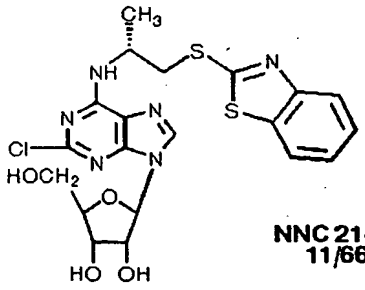
R-PIA
1.2/124/158



R = H, CHA
1.3/510/167
R = CH_3, **SDZ WAG 994**
76/5700/-



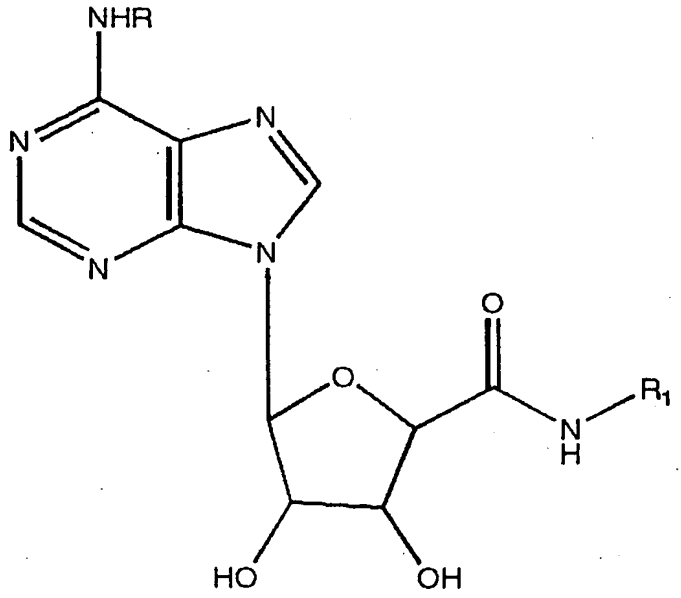
SPA
74/5700/526

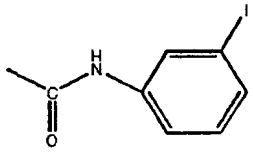
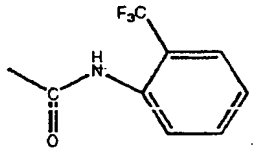
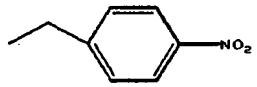
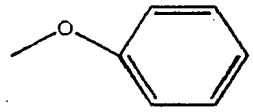
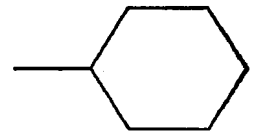


NNC 21-0136
11/660/-

EXAMPLE III
CARDIOPROTECTIVE EFFECT OF AGONISTS ACTIVATING BOTH A1 AND A3 RECEPTORS

In yet another embodiment of the invention, the administration of an agonist capable of activating both A1 and A3 receptors will be used in the methods of the invention for reducing ischemic damage to the heart. Referred to herein as a mixed A3/A1 agonist, these agents mediate superior cardioprotective effects. Examples of mixed A3/A1 agonists that may also be used in the practice of the present invention are listed in Table II.



COMPOUND (Reference)	R_1	R	A_1 (nm)	A_{2a} (nm)	A_3 (nm)
Compound 4q (Reference 27) MRS 646	Ethyl		16	3940	30
Compound 4d (Reference 27)	Ethyl		384	>10,000	54
Compound 37 (Reference 8)	Ethyl		49	574	9.0
Compound 11 (Reference 28)	Methyl		2060	66,300	1340
N^6-cyclohexyl NECA (Reference 29)	Ethyl		0.43	170	16

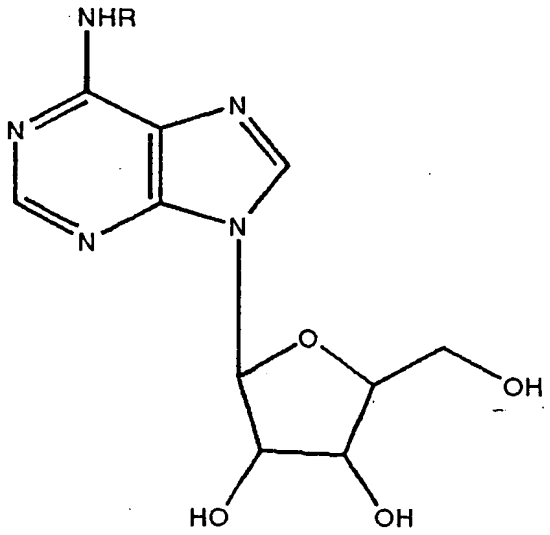
4q = N^6-((3-iodophenyl)carbamoyl)adenosine -5'uronamide

4d = N^6-((2-trifluoromethyl)carbamoyl)adenosine-5'uronamide

compound 37 = N^6-(4-Nitrobenzyl)adenosine-5'-N-ethyluronamide

compound 11 = 6-(O-Phenylhydroxylamino)purine-9-beta-ribofuranoside-5'-N-methyluronamide

N6-cyclohexyl NECA = N6-cyclohexyl 5'-N-ethylcarboxamidoadenosine

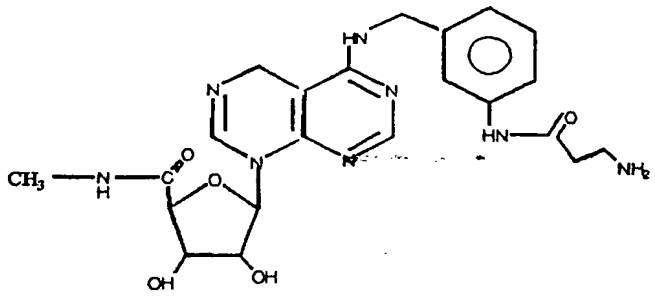


COMPOUND	R=	A_1 (nM)	A_2 (nM)	A_3 (nM)
Compound 8*	2-aminoethyl)amino] carbonyl]methyl]- anilino]carbonyl]methyl] phenyl	0.85	210	4

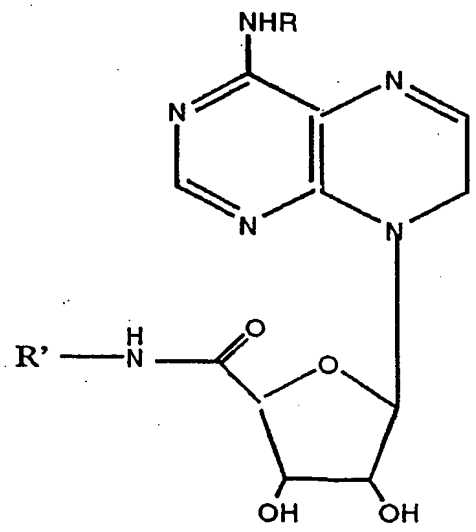
*N^6-[4-[[[4-[2-aminoethyl)amino]carbonyl]methyl]-anilino]carbonyl]methyl]phenyl]adenosine

Other examples of mixed A1/A3 agonists are MRS 580 and MRS 1364, the structures of which are shown below.

MRS 580



MRS 1364



R'=Et

R = CONH-phenyl-NO_2

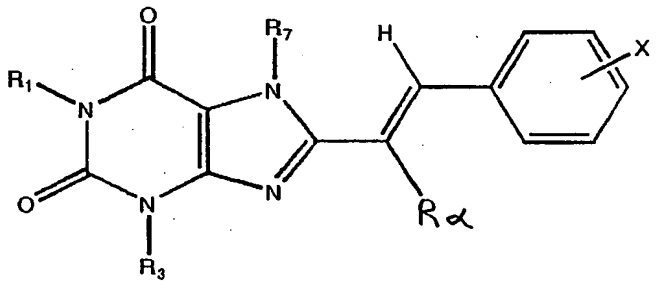
Figure 5A shows the results obtained when ventricular myocytes were exposed to the indicated concentrations of MRS646 (structure 4Q, table II) or MRS1364 for 5 minutes. Media were then replaced with fresh media, which was then followed by exposure to 90-minute simulated ischemia and the data graphed. The data show that these mixed A3/A1 agonists can pharmacologically induce preconditioning in cardiac myocytes.

Figure 5B shows that MRS 646 and MRS 1364 also potently protect against myocyte injury during the sustained simulated ischemia. Figures 5C and 5D show that these agonists can pharmacologically precondition the cardiac myocyte and induce potent cardioprotection via the human adenosine A3 receptor.

In yet another embodiment of the invention, a binary conjugate has also been synthesized which binds and activates both the A1 and A3 receptors simultaneously. Figure 6A shows that this binary conjugate, MRS1543, can pharmacologically precondition the cardiac myocyte and induce a potent cardioprotective effect. The protective effect is only partially blocked by A1 antagonist DPCPX; similarly, the protection is only partially attenuated by the A3 antagonist MRS1191. Figure 6B shows, however, that the combined presence of DPCPX and MRS1191 completely abolished the protective effect of MRS1543, indicating that the protection is mediated via activation of both the A1 and the A3 receptors. Figure 6C shows that MRS1543 can precondition and induces a cardioprotective effect via the human adenosine A3 receptor.

EXAMPLE IV
SIMULTANEOUS ADMINISTRATION OF A3/A1 ADENOSINE RECEPTOR AGONIST AND ADENOSINE A2a RECEPTOR ANTAGONIST GIVES RISE TO ENHANCED CARDIOPROTECTION

In yet another embodiment of the invention the simultaneous administration of A3 and A1 adenosine receptor agonist and A2a antagonist is contemplated. Preferred A2a adenosine receptor antagonists for use in the present invention are listed below in Table III:

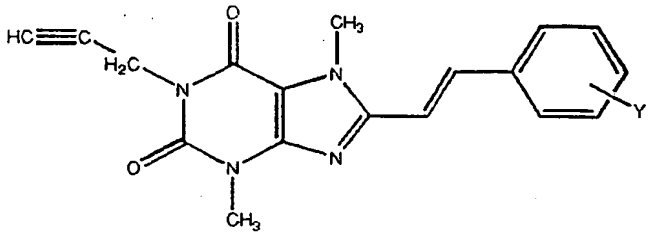


R1, R3 = methyl, ethyl, propyl, allyl
R7 = H, methyl, alkyl (C2-C8)
Rα = H (unless noted)
X = is shown in Table III

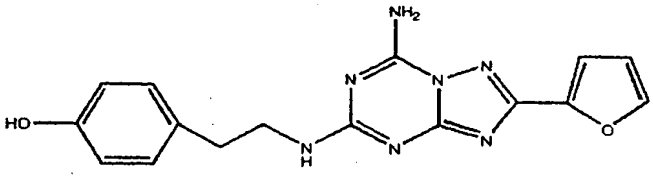
TABLE III
Affinities of 8 Styrylxanthine Derivatives in Radioligand Binding Assays at Rat Brain A_1 and A_2-Receptors (31)

cmpd	R_1,R_3	R_7	X	A_1/A_2 ratio
15b	Me	Me	H	41
17b	Me	Me	2-MeO	18
19b	Me	Me	3-MeO	64
20b	Me	Me	3-CF_3	25
21b	Me	Me	3-NO_2	11
22b	Me	Me	3-NH_2–	30
23	Me	Me	3-(AcNH)	240
24	Me	Me	3-(HOOC(CH_2)$_2$CONH)	250
25	Me	Me	3-(*t*-BOC-NH)	30
26	Me	Me	3-[*t*-BOC)$_2$N]	15
27b	Me	Me	3-F	190
28	Me	Me	3-Cl	520
29b	Me	Me	4-MeO	44
32b	Me	Me	3,4-(MeO)$_2$	70
33a	Me	H	3,5-(MeO)$_2$	25
33b	Me	Me	3,5-(MeO)$_2$	>200
34b	Me	Me	3,5-F_2	230
35	Me	Me	3,5-(MeO)$_2$-4-OH	19
36	Me	Me	4-AcO-3,5-(MeO)$_2$	93
37	Me	Me	4-(4-PhCH_2O)-3,5-(MeO)$_2$	30
38	Me	Me	4-(4-NH_2-BuO)-3,5 (MeO)$_2$	36
39	Me	Me	4-[4-(*t*BOC-NH)BuO]-3,5-(MeO)$_2$	42
40	Me	Me	4-(4-NH_2-*trans*-CH_2CH= $CHCH_2O$-3,5-(MeO)$_2$	28
41	Me	Me	4-(4-AcNH-*trans*-CH_2CH= $CHCH_2O$)-3,5-(MeO)$_2$	>50
42	Me	Me	4-(4-*t*-BOC-NH-*trans*-CH_2CH= $CHCH_2O$-3,5-(MeO)$_2$	>40
43b	Me	Me	2,3,4-(MeO)$_3$	34
44b	Me	Me	3,4,5-(MeO)$_3$	70 [>5600]
45b	Et	Me	3,4,5-(MeO)$_3$	34
46	allyl	Me	3,4,5-(MeO)$_3$	13 [>6700]
51b	Pr	Me	3-Cl	14
52b	Pr	Me	3,4-(MeO)$_2$	19 [190]
53b	Pr	Me	3,5-(MeO)$_2$	110

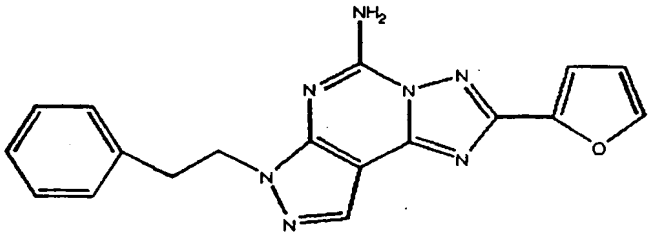
Additional compounds contemplated for use as A_{2a} antagonists include:



Y = m-Br or p-SO_3H (DMPX)



(ZM241385)



(SCH58261)

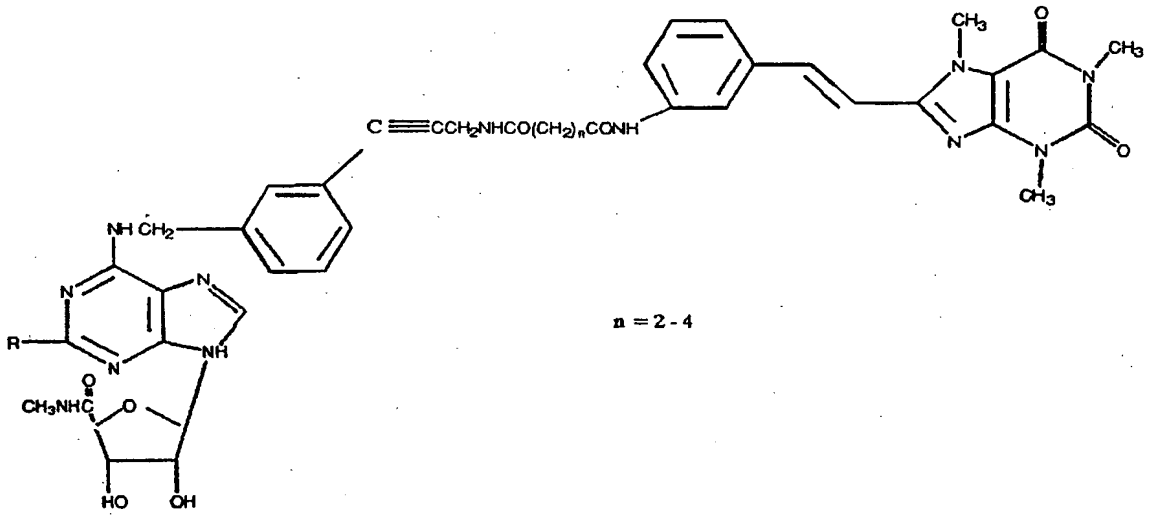
The results shown in Figures 7A and 7B indicate that the simultaneous administration of an A3/A1 agonist and an A2a antagonist gives rise to enhanced cardioprotection. Cardiac myocytes were prepared as described in Example I. The A1/A3 agonist, MRS 580, was delivered in the presence or absence of the A2a antagonist CSC. Data were plotted as the percentage of cells killed vs. the various drug combinations as indicated. Data represent the means ± S.E. of three experiments. Figure 7C shows that MRS 580 can precondition the cardiac myocytes via the human adenosine A3 receptor.

In another embodiment, a binary conjugate, MRS 1528, was synthesized which acts as an agonist at the A3 receptor and an antagonist at the A2a receptor simultaneously. Figure 8A shows that the protective effect of MRS1528 was unaffected by the A2a receptor antagonist CSC, consistent with the ability of MRS1528 to block the A2a receptor. Figure 8B shows that the protective effect of low concentrations of MRS1528 is attenuated by the A2a agonist CGS21680 whereas the protective effect of high concentrations of MRS1528 is not affected by the presence of CGS21680. Together, these data indicate that MRS1528 can activate A3 receptor to induce preconditioning and can simultaneously block the A2a receptor to enhance its preconditioning effect. Further support for this concept comes from studies testing the A3 agonist moiety of MRS1528, MRS1525. MRS1525 does not contain the A2a antagonist moiety and in response to the CSC, showed a uniform CSC-mediated increase in the extent of preconditioning effect (Fig. 8C). In the concomitant presence of CGS21680, the preconditioning effect of MRS1525 is attenuated at both the high and low concentrations of MRS1525 (Fig. 8D). Figure 8E shows that MRS1528 can cause preconditioning of

the cardiac myocytes via human adenosine A3 receptor.

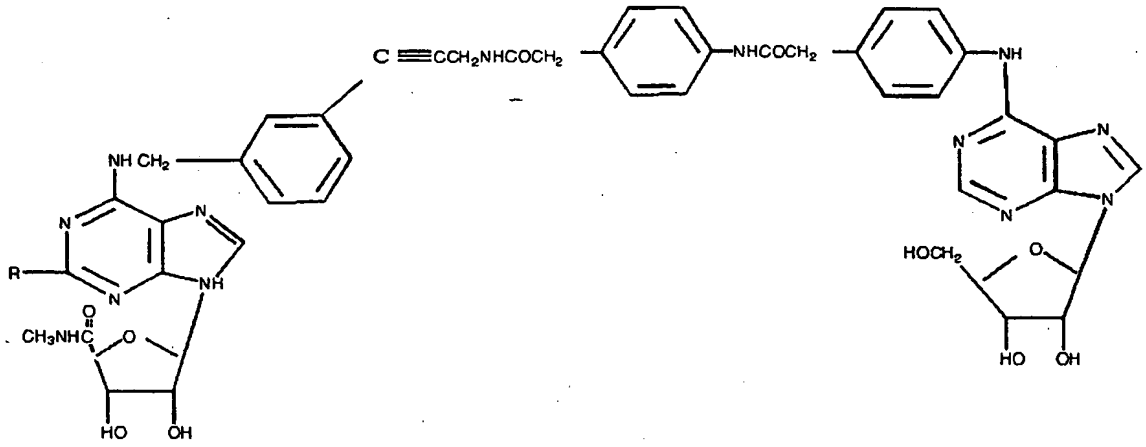
A binary conjugate was synthesized with the general structure shown below. This conjugate binds both the A2a and the A3 receptors and acts as an agonist at the A3 receptor and an antagonist at the A2a receptor simultaneously. In the exemplary conjugate disclosed herein, MRS 1528, R = H and n =2.

A3/A2a binary conjugate



A3/A2a Binary conjugate R = H, IB-MECA; R = Cl, Cl-IB-MECA

A second binary conjugate has also been synthesized which binds and activates both the A1 adenosine and A3 adenosine receptors simultaneously and has the general structure shown below. In an exemplary A1/A3 binary conjugate of the invention, MRS 1543, R=H.



A3/A1 binary conjugate
R = H, IB-MECA
R = Cl, CL-IB-MECA

Figure 9 sets forth an exemplary synthetic scheme utilized to produce the binary conjugate specific for the A1 and A3 receptors.

Figures 10A -10C depict schematic diagrams for synthesizing the compounds of the invention. Figures 10A-10B shows a synthetic scheme for generating a derivative of an A1 selective agonist for coupling to an amine derived A3 agonist. Figure 10C shows a synthetic scheme for conjugating the reagents via an extended linker. Extended linkers may increase the affinity and potency of the conjugates at the adenosine receptor. Table IV lists the names of the chemical structures appearing in Figures 9, 10B and 10C.

TABLE IV

IB-MECA = N^6-(3-iodobenzyl)-5′-N-methylcarboxamidoadenosine

Compound III = N^6-[3-(3-amino-1-propynyl)benzyl]-5′-N-methylcarboxamidoadenosine

Compound VIII (m- and p-isomers) = N^6-[3-[3-(3-isothiocyanatophenylaminothiocarbonyl)-amino-1-propynyl]benzyl]-5′-N-methylcarboxamidoadenosine and N^6-[3-[3-(4-isothiocyanatophenylaminothiocarbonyl)-amino-1-propynyl]benzyl]-5′-N-methylcarboxamidoadenosine

ADAC = N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]anilino]carbonyl]methyl]phenyl]adenosine

Compound IX (m- and p-isomers) = conjugate of N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]anilino]carbonyl]methyl]phenyl]adenosine and N^6-[3-[3-(3-isothiocyanatophenylaminothiocarbonyl)-amino-1-propynyl]benzyl]-5′-N-methylcarboxamidoadenosine and conjugate of N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]anilino]carbonyl]methyl]phenyl]adenosine and N^6-[3-[3-(4-isothiocyanatophenylaminothiocarbonyl)-amino-1-propynyl]benzyl]-5′-N-methylcarboxamidoadenosine

Compounds XI =

MRS 1576 = t-Boc-L-alanyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5′-N-methylcarboxamidoadenosine

MRS 1574 = t-Boc-L-methionyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5′-N-methylcarboxamidoadenosine

MRS 1568 = t-Boc-L-valyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5′-N-methylcarboxamidoadenosine

MRS 1571= t-Boc-L-leucyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5′-N-methylcarboxamidoadenosine

MRS 1572 = t-Boc-L-isoleucyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5′-N-

methylcarboxamidoadenosine

MRS 1573 = t-Boc-L-phenylalanyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5'-N-methylcarboxamidoadenosine

Compounds XII =

MRS 1577 = L-alanyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5'-N-methylcarboxamidoadenosine

MRS 1575 = L-methionyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5'-N-methylcarboxamidoadenosine

MRS 1570 = L-valyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5'-N-methylcarboxamidoadenosine

MRS 1569= L-leucyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5'-N-methylcarboxamidoadenosine

MRS 1560= L-isoleucyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5'-N-methylcarboxamidoadenosine

MRS 1565 = L-phenylalanyl-N^6-[3-(3-amino-1-propynyl)benzyl]-5'-N-methylcarboxamidoadenosine

Compound IV = N^6-[4-[[[4-(carboxymethyl)anilino]carbonyl]methyl]phenyl]adenosine

Compound XIII = conjugate of N^6-[4-[[[4-(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl]adenosine and compound XII

Compound XIV = N^6-[4-(carboxymethyl)phenyl]adenosine

Compound XV = conjugate of N^6-[4-(carboxymethyl)phenyl]adenosine and compound XII

EXAMPLE V
SCREENING ASSAY FOR IDENTIFYING COMPOUNDS THAT HAVE AFFINITY FOR THE A3 ADENOSINE RECEPTOR.

The recombinant cardiac atrial cells described above provide a system for assessing agents that may have cardioprotective activity. A generalized method for such screening would entail providing a fixed concentration of a test compound to transfected cells and assessing whether or not the test compound exerted a protective effect during ischemia. Negative controls would comprise both untranfected cells and transfected cells not exposed to the test compound.

Once a test compound is determined to have cardioprotective activity, it will be serially diluted and applied to the transfected myocytes described above. In this way, the minimally effective concentration of the compound will be determined.

While $CaPO_4$ transfection is exemplified herein, the myocytes of the invention may be transfected using any method known to those of skill in the art. Such methods include, but are not limited to lipofectin, electroporation, or viral vector mediated transfection.

Cardiac myocytes may be transfected with any of the adenosine receptor having a known DNA sequence. Thus the assay is not limited to cells transfected with A3 encoding cDNA. Any of the known adenosine receptors may be transfected and assayed as described above. Figure 11 shows the nucleotide sequence of the cDNA encoding the A1 adenosine receptor. Figure 12 shows the nucleotide sequence of the cDNA encoding the A2a receptor and Figure 13 shows the sequence of the cDNA encoding the A3 receptor (32-34).

EXAMPLE VI

ADMINSTRATION MODALITIES SUITABLE FOR THE COMPOUNDS OF THE PRESENT INVENTION

The protective effect of A3/A1 agonists has been demonstrated herein in animal models. A1/A3 agonists may be used therapeutically in patients who suffer from ischemic damage due to stable angina, unstable angina or post-myocardial infarction angina.

Several administration modalities may be utilized to treat patients with the agonists and antagonists of the invention. These modalities are influenced by bioavailability factors. For example, if the compound is metabolized in the liver or excreted in the bile, some of the active compound absorbed from the gastrointestinal tract will be inactivated by the liver before it can reach the general circulation for distribution to the site of action. It is not believed that the compounds of the invention will be subject to this first pass loss. Additionally, because the agonists of the invention are polar and water soluble, it is expected that they will have a small volume of distribution, and thus be readily eliminated by the kidney. Moreover, binding of the agonists to plasma proteins may limit their free concentrations in tissues and at their locus of action since it is only the unbound drug which equilibrates across membrane receptor sites.

Another factor affecting bioavailability is the distribution of the agonists to tissues. The agonists of the invention do not cross the blood brain barrier. Given the relatively small size of the compounds and their water solubility, it is anticipated that the compounds will have a relatively fast second phase of drug distribution. This distribution is determined by both the blood flow to the particular tissue of the organ such as the heart, as well as the rate at which the compounds diffuse into the interstitial compartment from

the general circulation through the highly permeable capillary endothelium.

Patients may be perfused with the agonists of the invention by dissolving them in normal saline solution or using emulsifying agents or cosolvents followed by intravenous administration every four to six hours. Effective doses usually range from 100 to 300 nM. For example, considering a 15 liter volume of distribution for a 70 kg patient, a loading dose ranging from 0.5 to 1.5 mg is preferably used. Depending on the half-life of the agonists in the body, several doses, e.g., 1.5-4.5 mg may be adminstered per day.

Alternatively, a time-release or slow-release preparation may be utilized which allows for periodic or constant release of the antagonists over a given time period. This method would allow for a single dose of the agonists in a given day. Methods for preparing such capules are well known to those of skill in the art of drug delivery.

In summary, the present data illustrates the novel synergistic protective function of simultaneous cardiac A1 and A3 receptor activation. In addition to the synergistic role in mediating preconditioning of the cardiac myocyte, the data provide conclusive evidence that activation of both receptors can also act synergistically to attenuate myocyte injury during the prolonged injury-producing ischemia. Thus, agonists selective at the A1 and the A3 receptors represent novel potent cardioprotective agents even when ischemia has already begun. The concomitant administration of A1/A3 agonist with an A2a antagonist may also enhance cardioprotection as will binary conjugate capable of activating the A1 or A3 receptor while simultaneously blocking the A2a receptor. These data have important clinical implications in the treatment of ischemic heart disease and implicate A1 and A3 receptor-selective agonists for the reduction of the size of myocardial

infarction when given during the infarct-producing ischemia.

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While certain preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made to the invention without departing from the scope and spirit thereof as set forth in the following claims.

What is claimed is:

1. A method for preventing or reducing ischemic damage to the heart, in a patient in need thereof, comprising administering to said patient an agonist having affinity for both the A1 and A3 adenosine receptors in an amount effective to activate A3 and A1 receptors in the heart of said patient.

2. A method as claimed in claim 1, wherein said agonist is delivered using an administration means selected from the group consisting of intravenous administration, oral administration and cardiac perfusion.

3. A method as claimed in claim 1, wherein said agonist is selected from the group of compounds listed in Table II.

4. A method as claimed in claim 1, wherein said agonist is N^6-((2-trifluoromethyl)carbamoyl) adenosine-5'uronamide.

5. A method as claimed in claim 1, wherein said agonist is N^6-((3-iodophenyl)carbamoyl) adenosine-5'uronamide.

6. A method as claimed in claim 1 wherein said agonist is a binary conjugate which has affinity for, and activates the A1 and A3 adenosine receptors simultaneously.

7. A method as claimed in claim 1 wherein said agonist is administered to said patient prior to a surgical procedure having potential to cause cardiac ischemic damage.

8. A method as claimed in claim 1, wherein said agonist is administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

9. A method as claimed in claim 1, wherein said agonist is administered to said patient following a surgical procedure having potential to result in cardiac ischemic damage.

10. A method as claimed in claim 1, wherein said patient is in need of said treatment due to an anginal condition selected from the group consisting of chronic stable angina, unstable angina, post myocardial infarction angina.

11. A method as claimed in claim 1, wherein said patient is in need of such treatment due to acute myocardial infarction.

12. A method for preventing or reducing ischemic damage to the heart, in a patient in need thereof, comprising administering to said patient an mixed agonist having affinity for the A3 and A1 adenosine receptors and an antagonist having affinity for the A2a adenosine receptor in amounts effective to activate said A3 and A1 receptors and inhibit activation of said A2a receptor in the heart of said patient.

13. A method as claimed in claim 12, wherein said agonist and said antagonist are delivered using an administration means selected from the group consisting of intravenous administration, oral administration and cardiac perfusion.

14. A method as claimed in claim 12, wherein said agonist is selected from the group of compounds listed in

Table II.

15. A method as claimed in claim 12 wherein said antagonist is selected from the group of compounds listed in Table III.

16. A method as claimed in claim 12, wherein said agonist is N^6-((2-trifluoromethyl)carbamoyl) adenosine-5'uronamide.

17. A method as claimed in claim 12, wherein said agonist is N^6-((3-iodophenyl)carbamoyl)adenosine-5'uronamide.

18. A method as claimed in claim 12, wherein said agonist is selected from the group consisting of MRS 584, MRS 479, MRS 537 or MRS 1340.

19. A method as claimed in claim 12, wherein said antagonist is selected from the group consisting of CSC, DMPX, ZM241385 or SCH58261.

20. A method as claimed in claim 12, wherein said agonist and said antagonist are administered to said patient prior to a surgical procedure having potential to cause cardiac ischemic damage.

21. A method as claimed in claim 12, wherein said agonist and said antagonist are administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

22. A method as claimed in claim 12, wherein said agonist and said antagonist are administered to said patient following a surgical procedure having potential to result in cardiac ischemic damage.

23. A method as claimed in claim 12, wherein said patient is in need of said treatment due to an anginal condition selected from the group consisting of chronic stable angina, unstable angina, and post myocardial infarction angina.

24. A method as claimed in claim 12, wherein said patient is in need of said treatment due to acute myocardial infarction.

25. A method for preventing or reducing ischemic damage to the heart, in a patient in need thereof, comprising administering to said patient a binary conjugate, which acts as an adenosine A3 receptor agonist while simultaneously inhibiting the activation of A2a receptors in an amount effective to enhance myocardial response to said preconditioning stimuli.

26. A method as claimed in claim 25, wherein said patient is in need of such treatment due to a cardiac condition selected from the group consisting of chronic stable angina, unstable angina, post-myocardial infarction angina or acute myocardial infarction.

27. A method as claimed in claim 25 wherein said agonist is administered to said patient prior to a surgical procedure which may cause cardiac ischemic damage.

28. A method as claimed in claim 25, wherein said agonist is administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

29. A method as claimed in claim 25, wherein said agonist is administered to said patient following a surgical procedure which may result in cardiac ischemic

damage.

30. A method for preventing or reducing ischemic damage to the heart, in a patient in need thereof, comprising administering to said patient both an adenosine A3 receptor agonist and at least one adenosine A1 receptor agonist in an amount effective to activate the A1 and A3 adenosine receptors in the heart of said patient.

31. A method as claimed in claim 30, wherein said agonists are delivered using an administration means selected from the group consisting of intravenous administration, oral administration and cardiac perfusion.

32. A method as claimed in claim 30, wherein said agonist and said agonists are administered to said patient prior to a surgical procedure having potential to cause cardiac ischemic damage.

33. A method as claimed in claim 30, wherein said agonist and said antagonist are administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

34. A method as claimed in claim 30, wherein said agonist and said antagonist are administered to said patient following a surgical procedure having potential to result in cardiac ischemic damage.

35. A method as claimed in claim 30, wherein said patient is in need of said treatment due to an anginal condition selected from the group consisting of chronic stable angina, unstable angina, and post myocardial infarction angina.

36. A method as claimed in claim 30, wherein said patient is in need of said treatment due to acute myocardial infarction.

37. A method as claimed in claim 30, wherein said A3 agonist is selected from the group of compounds consisting of IB-MECA, Cl-IB-MECA, MRS 584, MRS 479, MRS 537, MRS 1340 and DBXMR and said A1 agonist is selected from the group of compounds listed in Table I.

38. A binary conjugate for preventing or reducing ischemic damage to the heart, said conjugate acting as an agonist at the A3 adenosine receptor and an antagonist at the A2a adenosine receptor.

39. A binary conjugate as claimed in claim 38, said conjugate having the structure of MRS 1528.

40. A method for administering the binary conjugated of claim 38, wherein said conjugate is delivered using an administration means selected from the group consisting of intravenous administration, oral administration and cardiac perfusion.

41. A method as claimed in claim 40, wherein said conjugate is administered to said patient prior to a surgical procedure having potential to cause cardiac ischemic damage.

42. A method as claimed in claim 40, wherein said conjugate is administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

43. A method as claimed in claim 40, wherein said conjugate is administered to said patient following a

surgical procedure having potential to result in cardiac ischemic damage.

44. A method as claimed in claim 40, wherein said patient is in need of said treatment due to an anginal condition selected from the group consisting of chronic stable angina, unstable angina, and post myocardial infarction angina.

45. A method as claimed in claim 40, wherein said patient is in need of said treatment due to acute myocardial infarction.

46. A binary conjugate for preventing or reducing ischemic damage to the heart, said conjugate acting as an agonist at the A3 adenosine receptor and an agonist at the A1 adenosine receptor.

47. A binary conjugate as claimed in claim 46, said conjugate being selected from the group of compounds consisting of MRS1543, a conjugate of N^6-[4-[[[4-(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl] adenosine and compound XII of Figure 10B, wherein R′ on compound XII is H, a conjugate of N^6-[4-[[[4-(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl] adenosine and compound XII of Figure 10B, wherein R′ on compound XII is Ch_3, a conjugate of N^6-[4-[[[4-(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl] adenosine and compound XII of Figure 10B, wherein R′ is $(CH_2)_2SCH_3$, a conjugate of N^6-[4-[[[4-(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl] adenosine and compound XII of Figure 10B, wherein R′ is $CH(CH_3)_2$, a conjugate of N^6-[4-[[[4-(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl] adenosine and compound XII of Figure 10B, wherein R′ is $CH_2CH(CH_3)_2$, a conjugate of N^6-[4-[[[4-

(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl] adenosine and compound XII of Figure 10B, wherein R′ is $C(CH_3)CH_2CH_3$, and a conjugate of N^6-[4-[[[4-(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl] adenosine and compound XII of Figure 10B, wherein R′ is $CH_2C_6H_5$.

48. A binary conjugate as claimed in claim 46, said conjugate being selected from the group of compounds consisting of a conjugate of N^6-[4-(carboxymethyl) phenyl]adenosine and compound XII of Figure 10B, wherein R′ is H, a conjugate of N^6-[4-(carboxymethyl) phenyl]adenosine and compound XII of Figure 10B, wherein R′ is CH_3, a conjugate of N^6-[4-(carboxymethyl) phenyl]adenosine and compound XII of Figure 10B, wherein R′ is $(CH_2)_2SCH_3$, a conjugate of N^6-[4-(carboxymethyl) phenyl]adenosine and compound XII of Figure 10B, wherein R′ is $CH(CH_3)_2$, a conjugate of N^6-[4-(carboxymethyl) phenyl]adenosine and compound XII of Figure 10B, wherein R′ is $CH_2CH(CH_3)_2$, a conjugate of N^6-[4-(carboxymethyl) phenyl]adenosine and compound XII of Figure 10B, wherein R′ is $C(CH_3)CH_2CH_3$, and a conjugate of N^6-[4-(carboxymethyl) phenyl]adenosine and compound XII of Figure 10B, wherein R′ is $CH_2C_6H_5$.

49. A method for administering the binary conjugated of claim 46, wherein said conjugate is delivered using an administration means selected from the group consisting of intravenous administration, oral administration and cardiac perfusion.

50. A method as claimed in claim 49, wherein said conjugate is administered to said patient prior to a surgical procedure having potential to cause cardiac ischemic damage.

51. A method as claimed in claim 49, wherein said conjugate is administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

52. A method as claimed in claim 49, wherein said conjugate is administered to said patient following a surgical procedure having potential to result in cardiac ischemic damage.

53. A method as claimed in claim 49, wherein said patient is in need of said treatment due to an anginal condition selected from the group consisting of chronic stable angina, unstable angina, and post myocardial infarction angina.

54. A method as claimed in claim 49, wherein said patient is in need of said treatment due to acute myocardial infarction.

55. A recombinant cardiac myocyte comprising a nucleic acid encoding an adenosine receptor selected from the group consisting of the A3 receptor, the A3 receptor, or the A2a adenosine receptor.

56. A recombinant myocyte as claimed in claim 55, whrerein the myocyte is a chick embryo ventricular myocyte and the adenosine receptor is a human adenosine receptor.

57. A method for determining whether a test compound exerts a cardioprotective effect, comprising:

a) providing a recombinant myocyte expressing an adenosine receptor;

b) contacting said cells with said test compound;

c) exposing cells to ischemic conditions; and

d) assessing the presence of said cardioprotective effect, if any, exerted by said test compound.

58. A method as claimed in claim 57, wherein said cardioprotective effect is assessed by determining the number of myocytes killed.

59. A method as claimed in claim 57, wherein said cardioprotective effect is assessed by determining the amount of creatine kinase released from said recombinant cardiac myocytes.

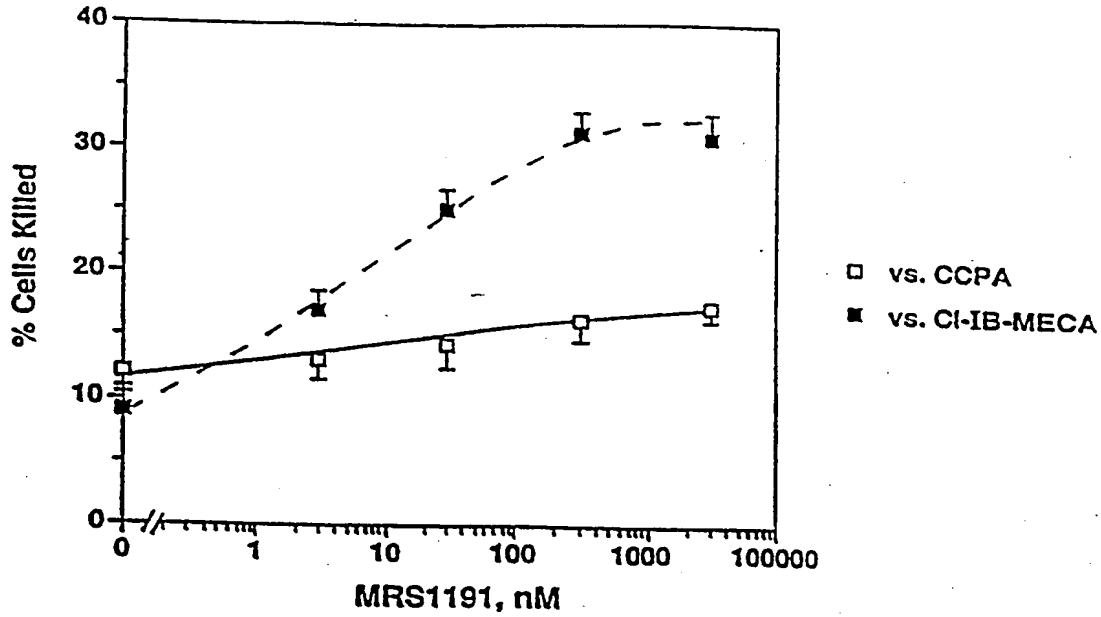
60. A method as claimed in claim 57, wherein said recombinant myocyte is selected from the group consisting of chick embryo ventricular myocytes or adult rat ventricular myocytes.

61. A method as claimed in claim 57, wherein said adenosine receptor is the human A3 adenosine receptor.

62. A method as claimed in claim 57, wherein said adenosine receptor is the human A1 adenosine receptor.

63. A method as claimed in claim 57, wherein said adenosine receptor is the human A2a adenosine receptor.

Fig. 1A


40
30
20
10
0
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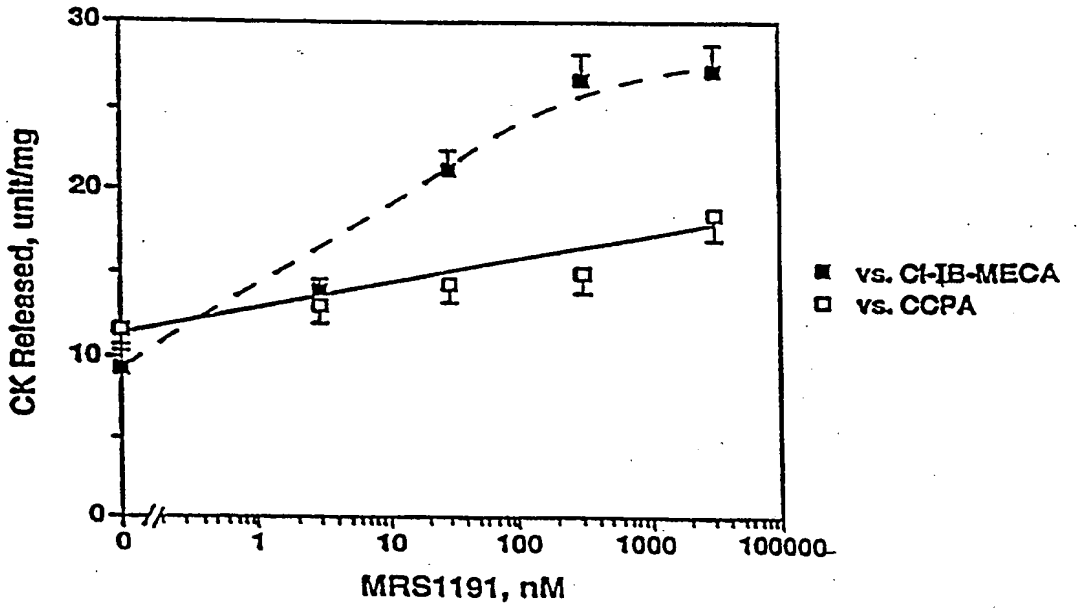
0
1
10
100
1000
100000
MRS1191, nM
vs. CCPA
vs. Cl-IB-MECA

Fig. 1B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20
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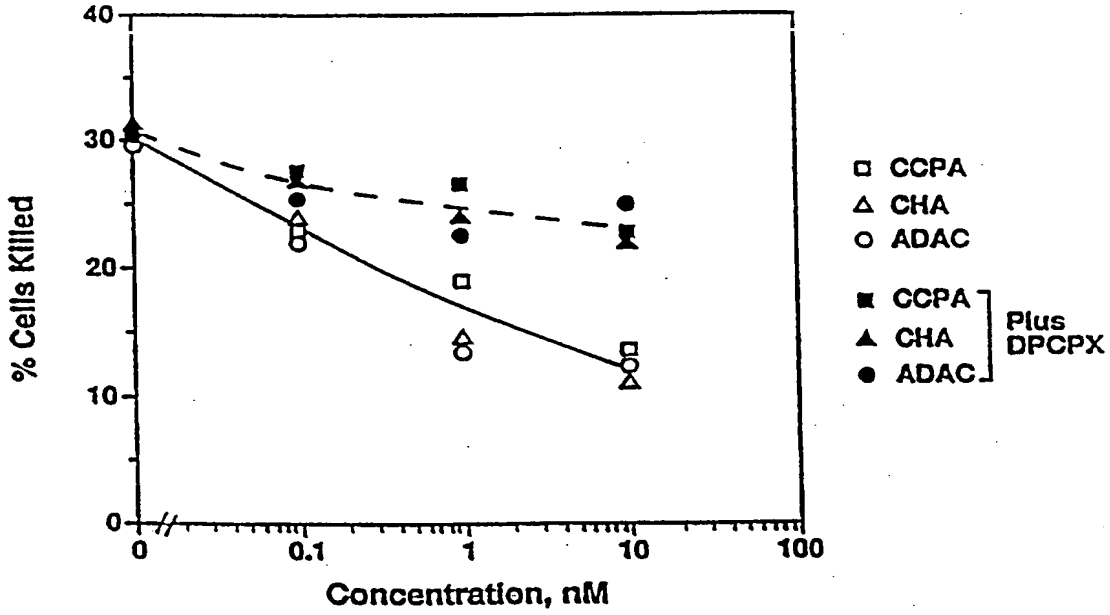
1
10
100
1000
100000
MRS1191, nM
vs. Cl-IB-MECA
vs. CCPA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30
20
10
0
% Cells Killed
0
0.1
1
10
100
Concentration, nM
CCPA
CHA
ADAC
CCPA
CHA
ADAC
Plus
DPCPX

Figure 2

Fig. 3A

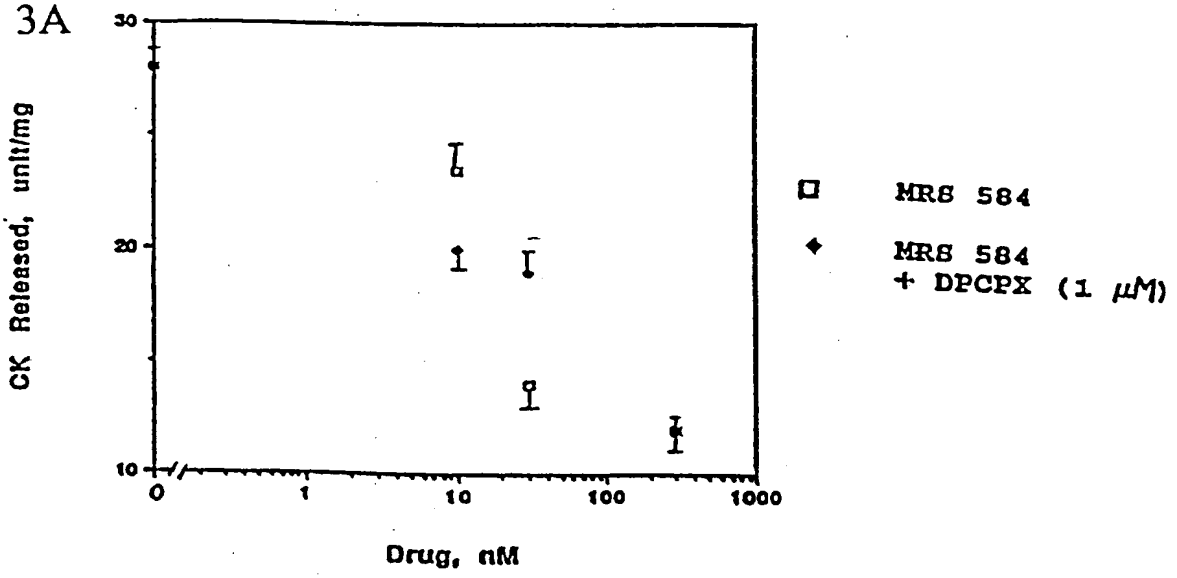


Fig. 3B

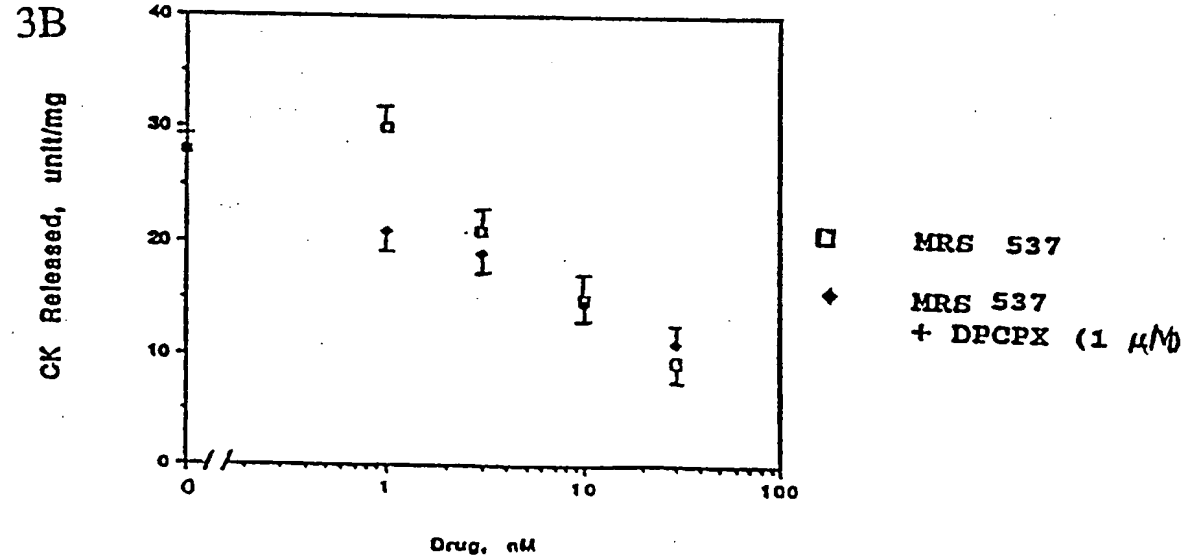
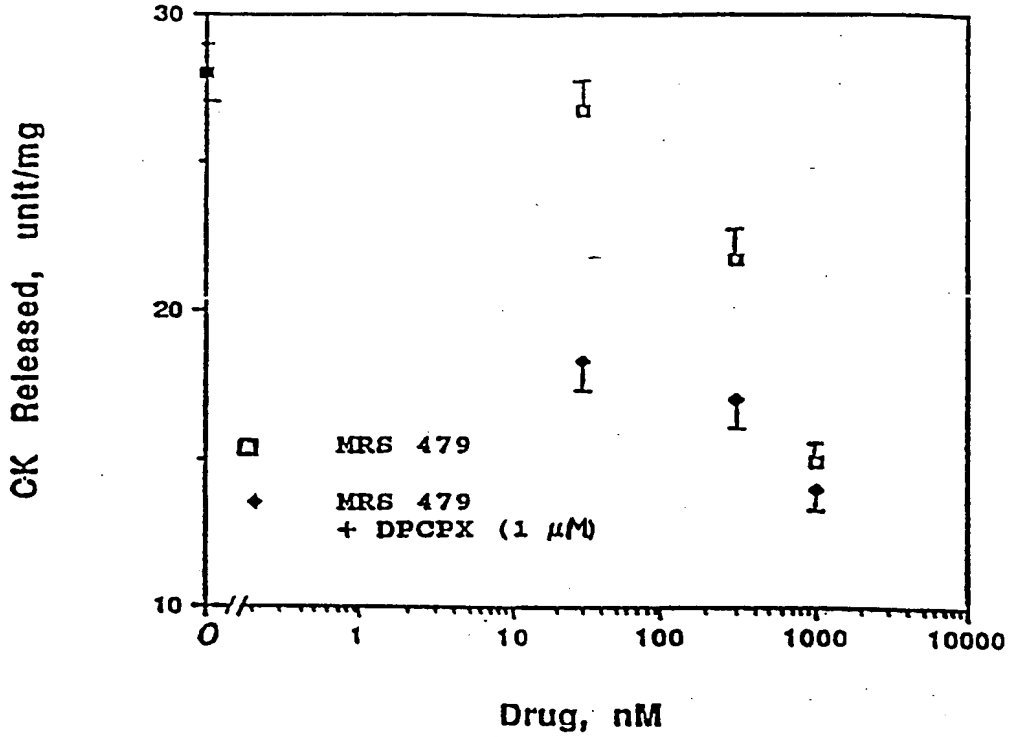


Fig. 3C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20
10
CK Released, unit/mg
MRS 479
MRS 479
+ DPCPX (1 μM)
0
1
10
100
1000
10000
Drug, nM

Fig. 3D

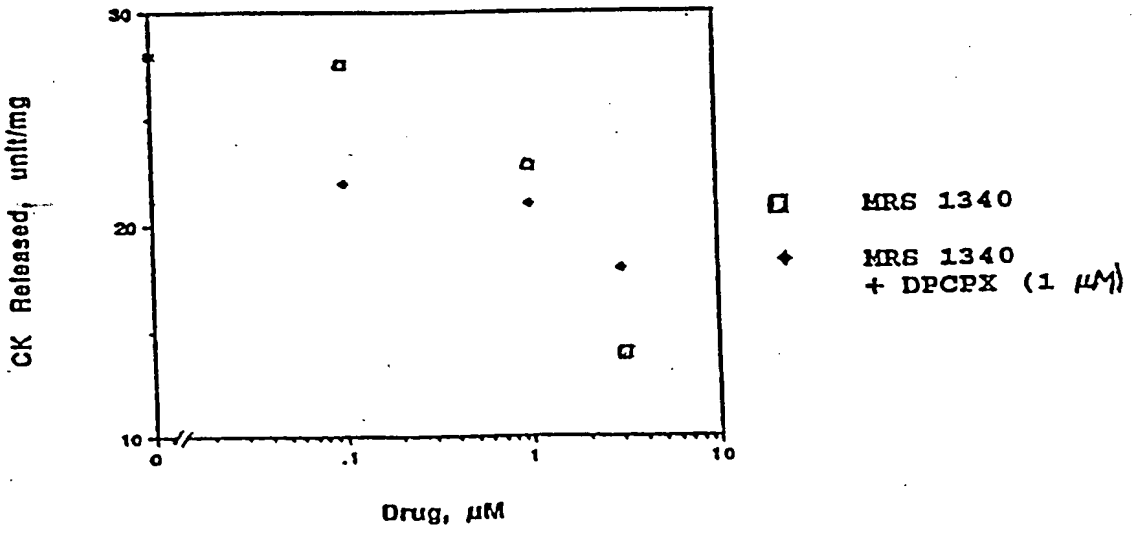

30
20
10
CK Released, unit/mg
0
.1
1
10
Drug, μM
MRS 1340
MRS 1340
+ DPCPX (1 μM)

Fig. 3E

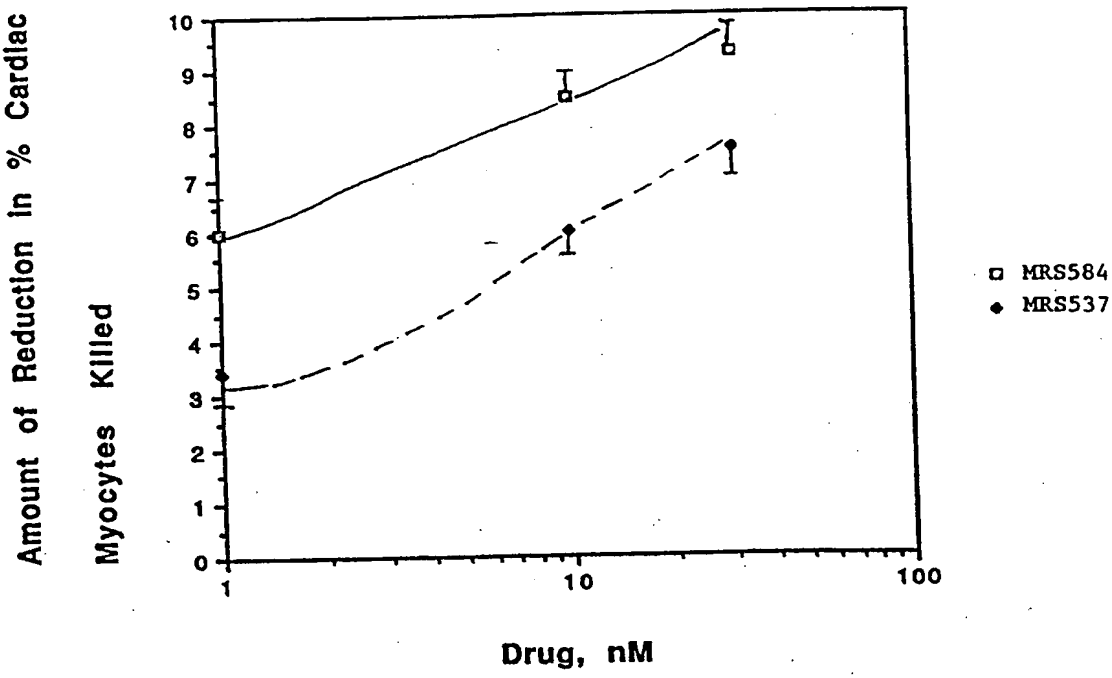


Fig. 3F

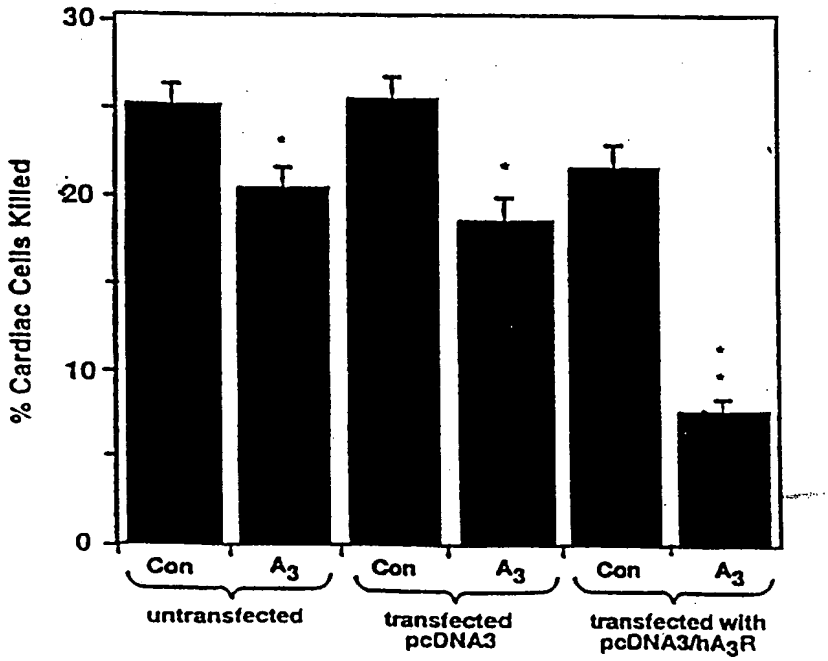


Fig. 4A

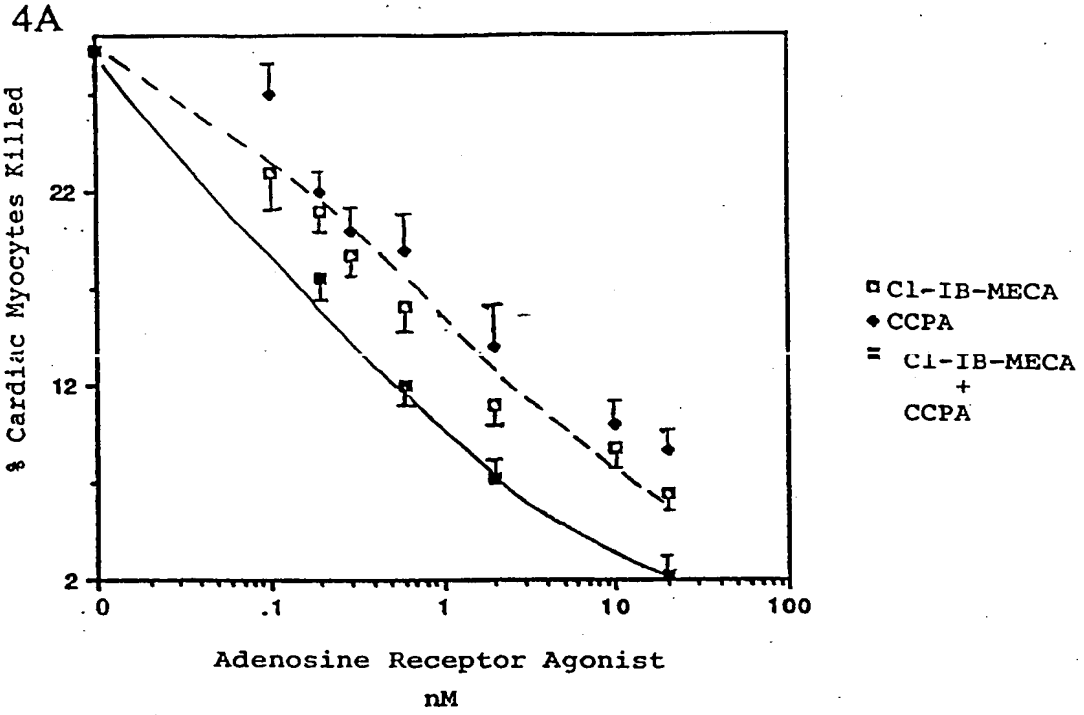

% Cardiac Myocytes Killed
22
12
2
0
.1
1
10
100
Adenosine Receptor Agonist
nM
Cl-IB-MECA
CCPA
Cl-IB-MECA + CCPA

Fig. 4B

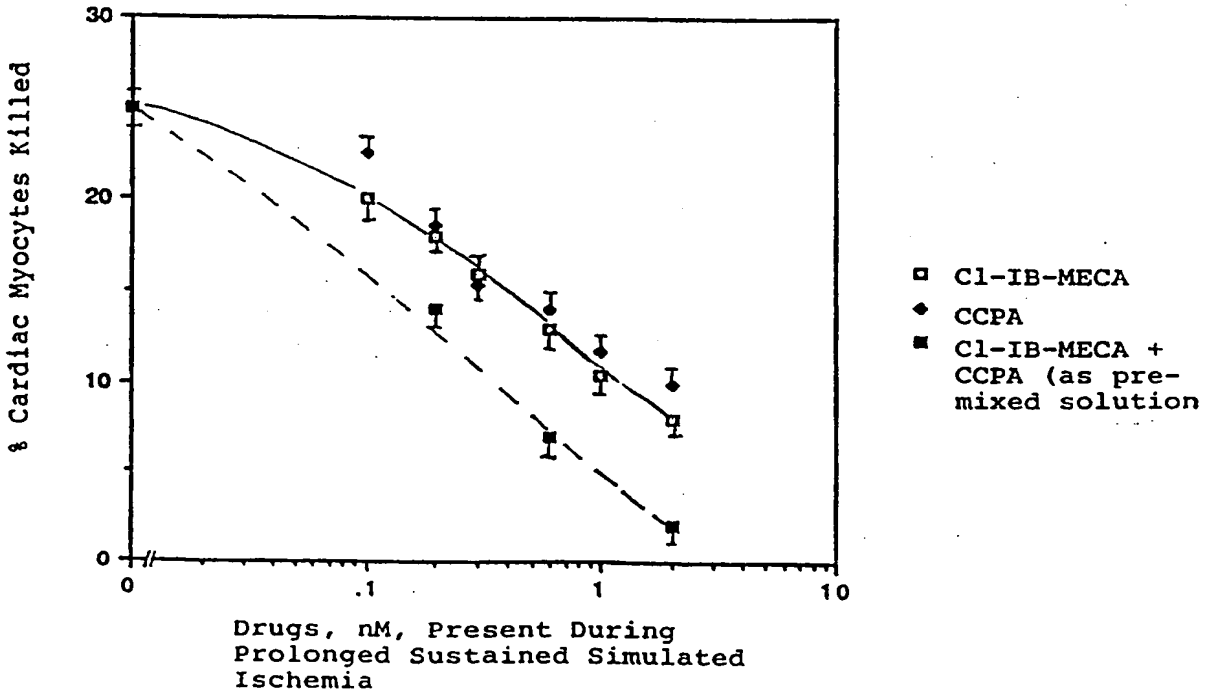

% Cardiac Myocytes Killed
30
20
10
0
0
.1
1
10
Drugs, nM, Present During Prolonged Sustained Simulated Ischemia
Cl-IB-MECA
CCPA
Cl-IB-MECA + CCPA (as pre-mixed solution

Fig. 5A

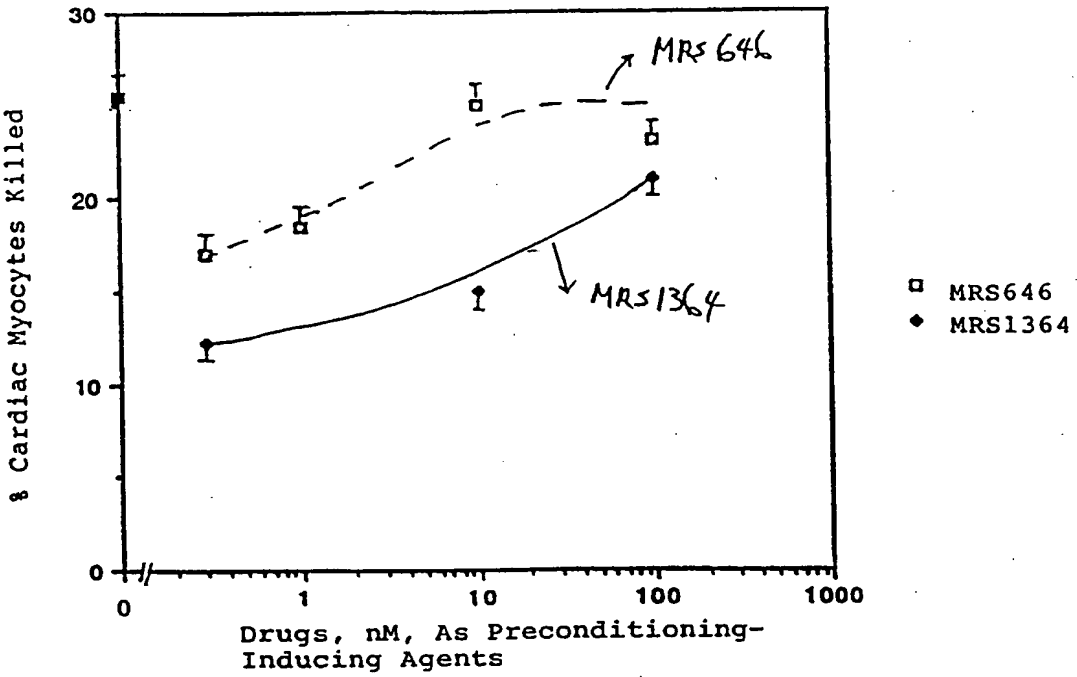


Fig. 5B

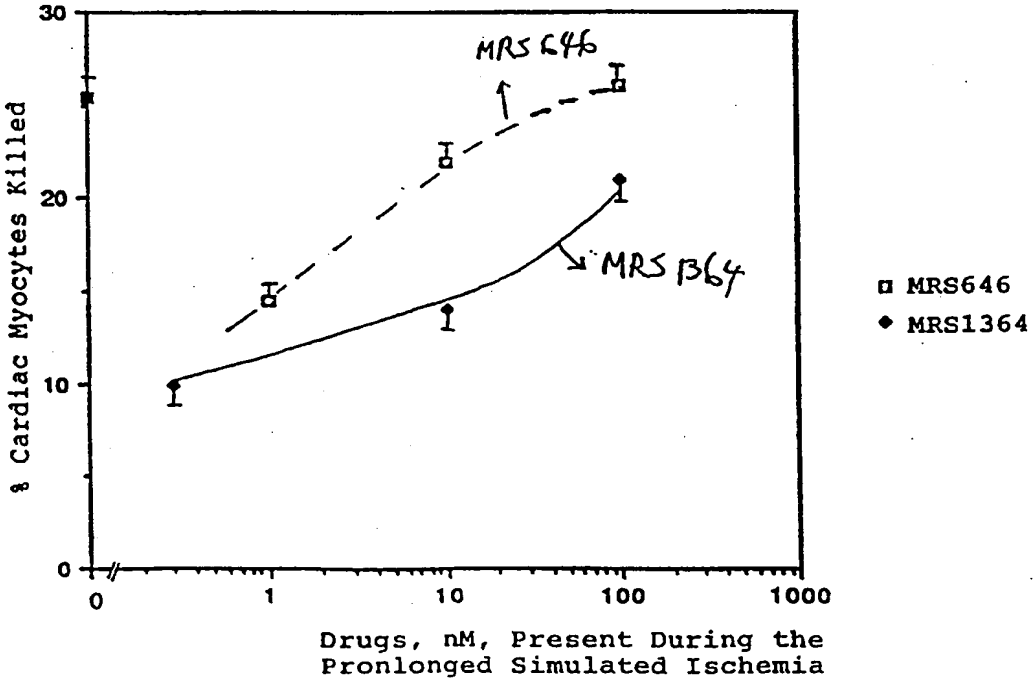


Fig. 5C

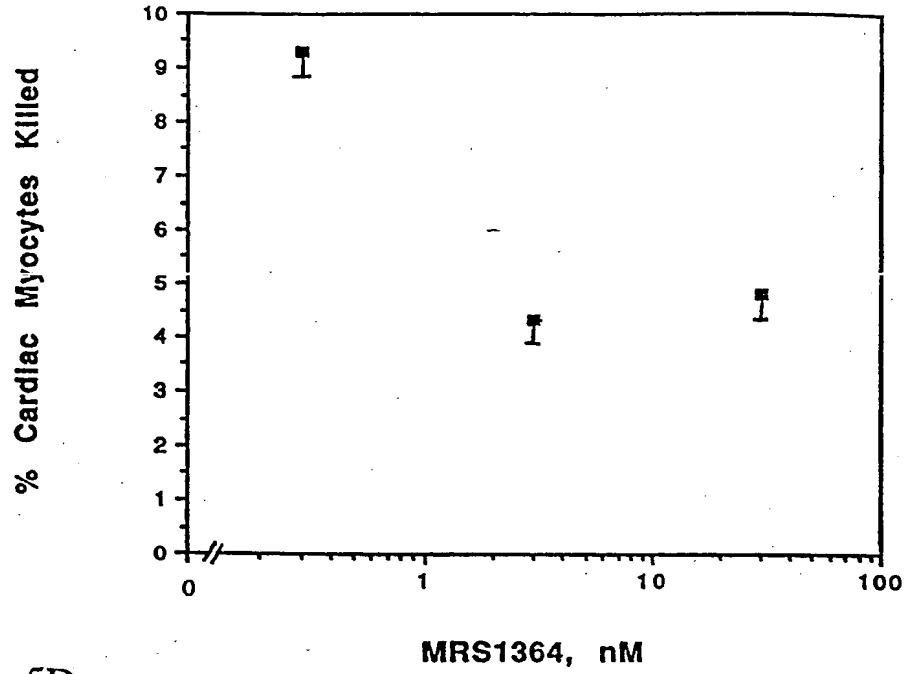

% Cardiac Myocytes Killed
10
9
8
7
6
5
4
3
2
1
0
0
1
10
100
MRS1364, nM

Fig. 5D

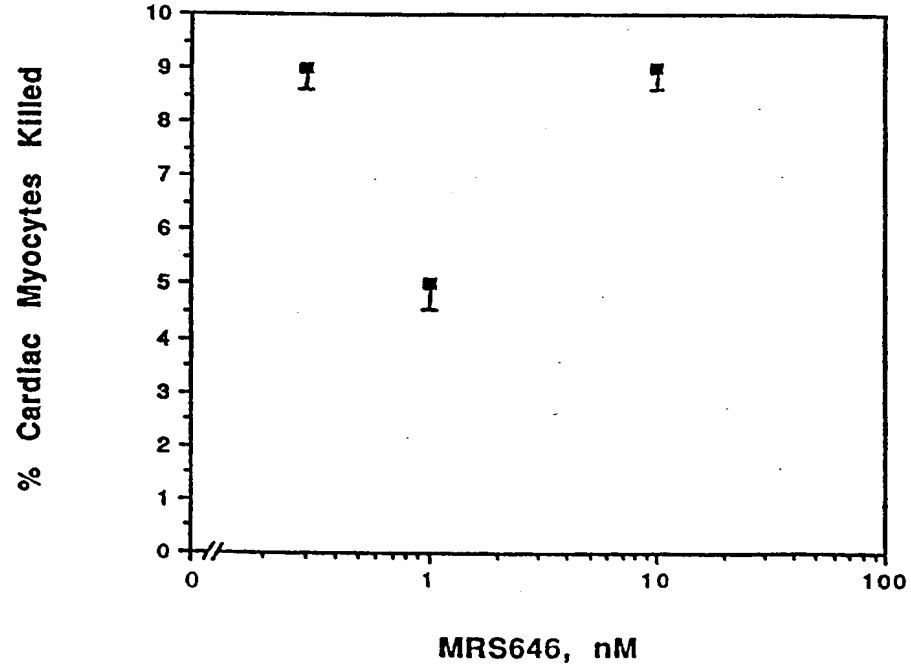

% Cardiac Myocytes Killed
10
9
8
7
6
5
4
3
2
1
0
0
1
10
100
MRS646, nM

Fig. 6A

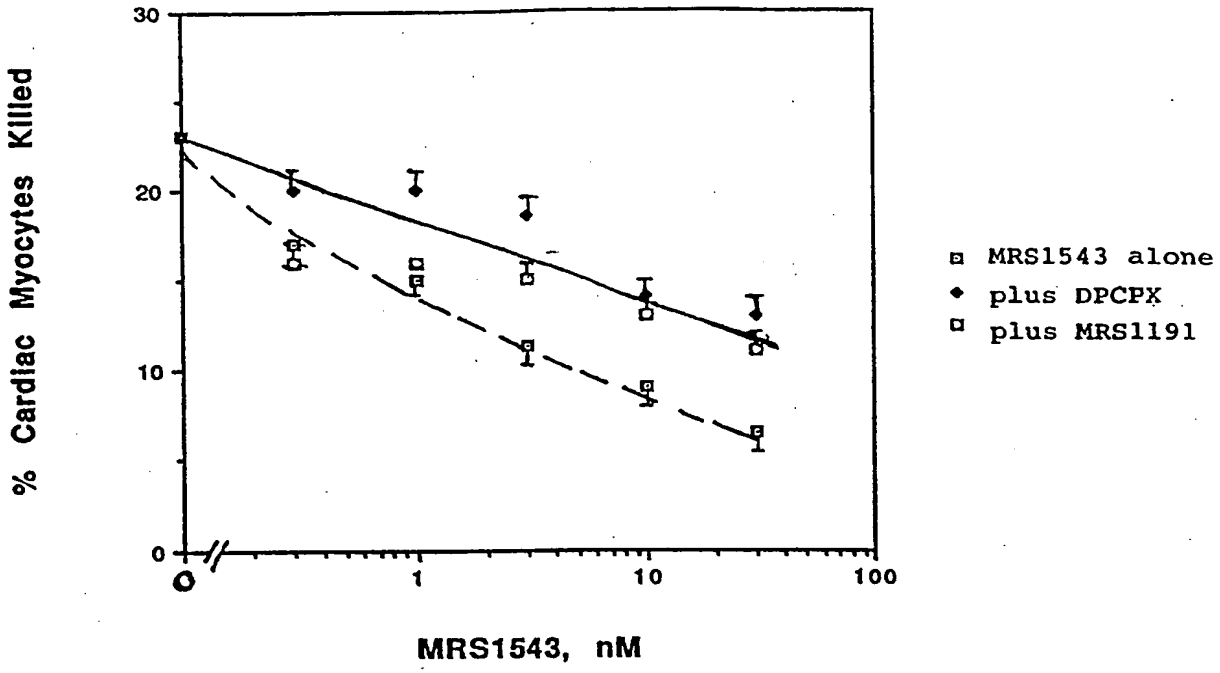


Fig. 6B

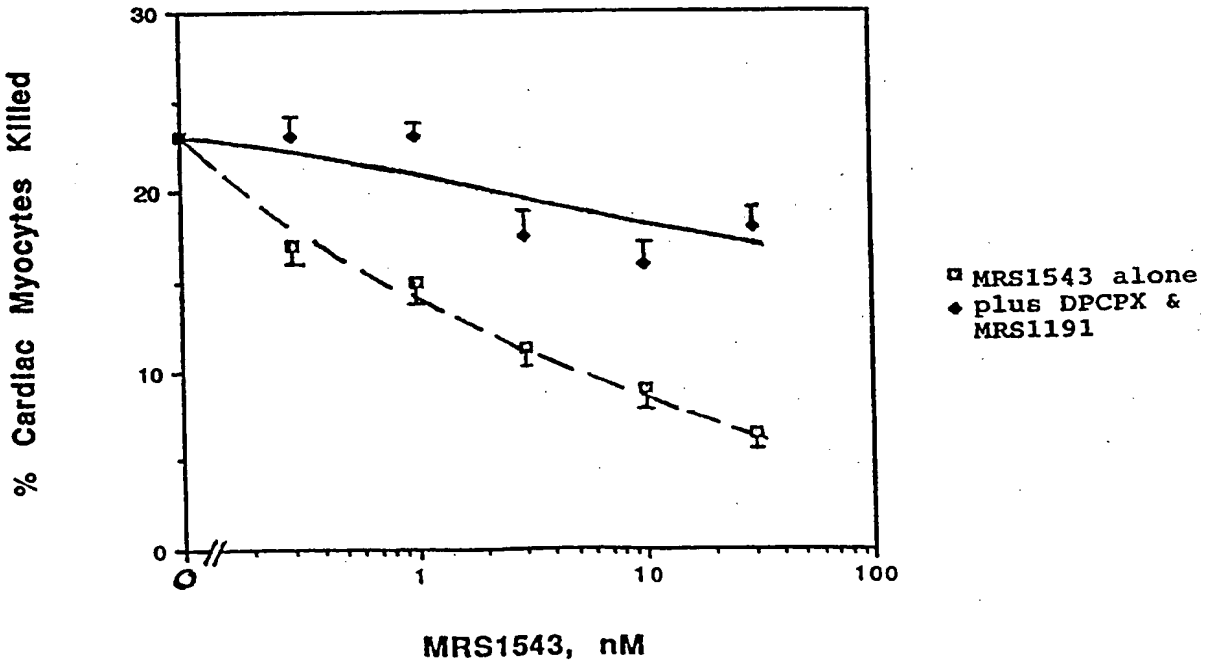


Fig. 6C

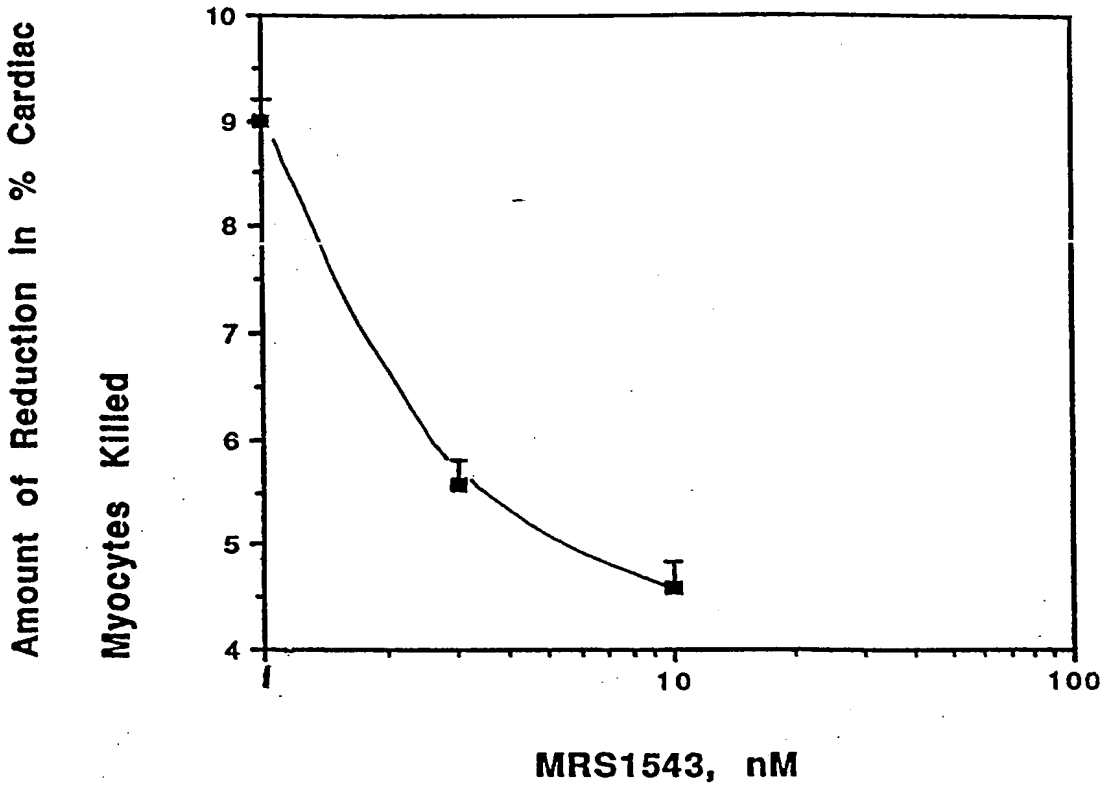

Amount of Reduction In % Cardiac Myocytes Killed
10
9
8
7
6
5
4
1
10
100
MRS1543, nM

Fig. 7A

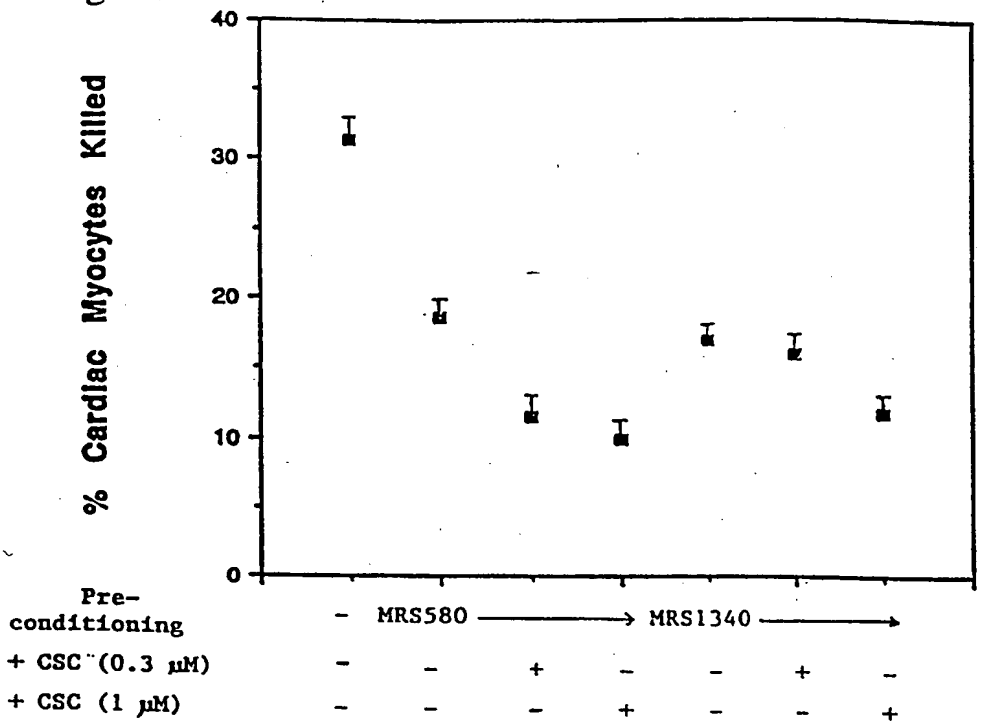

% Cardiac Myocytes Killed
40
30
20
10
0
Pre-conditioning
– MRS580 → MRS1340
+ CSC (0.3 μM)
+ CSC (1 μM)

Fig. 7B

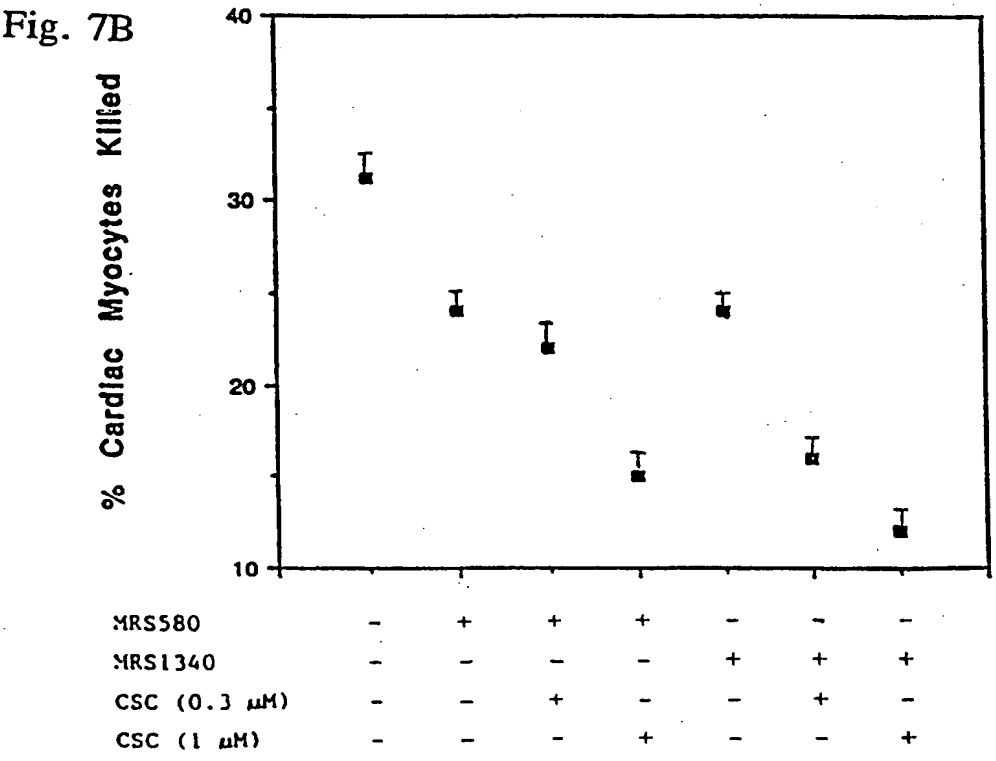

% Cardiac Myocytes Killed
40
30
20
10
MRS580
MRS1340
CSC (0.3 μM)
CSC (1 μM)

Fig. 7C

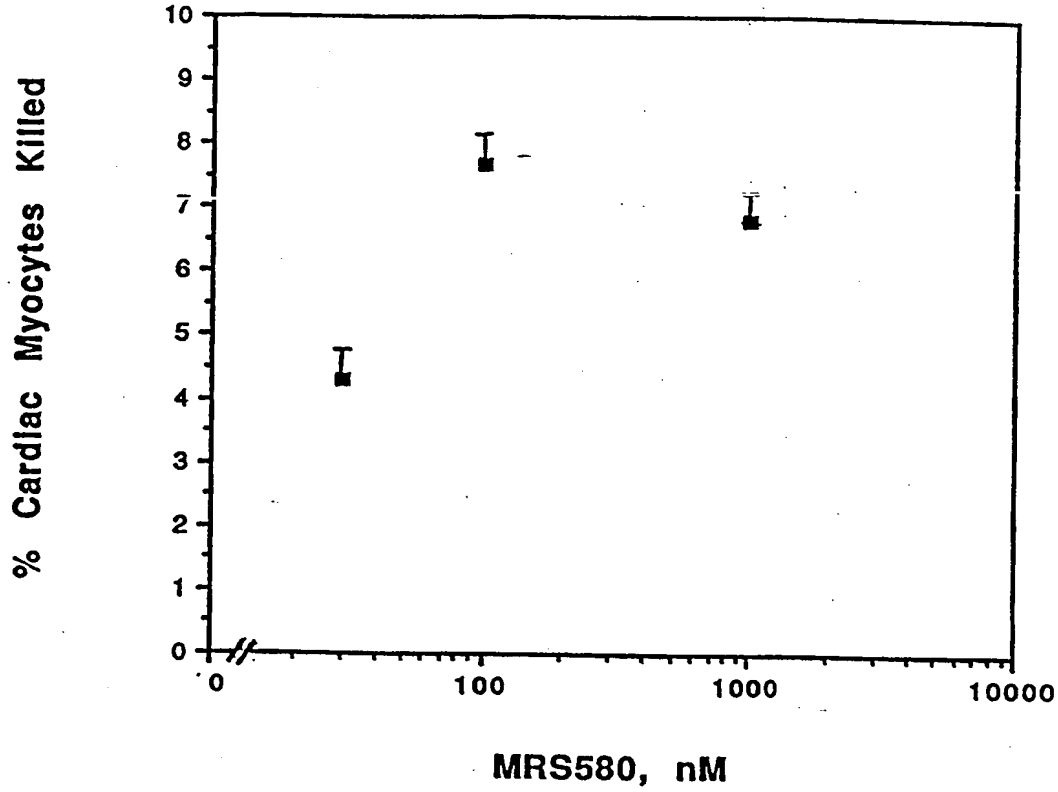

% Cardiac Myocytes Killed
10
9
8
7
6
5
4
3
2
1
0
0
100
1000
10000
MRS580, nM

Fig. 8A

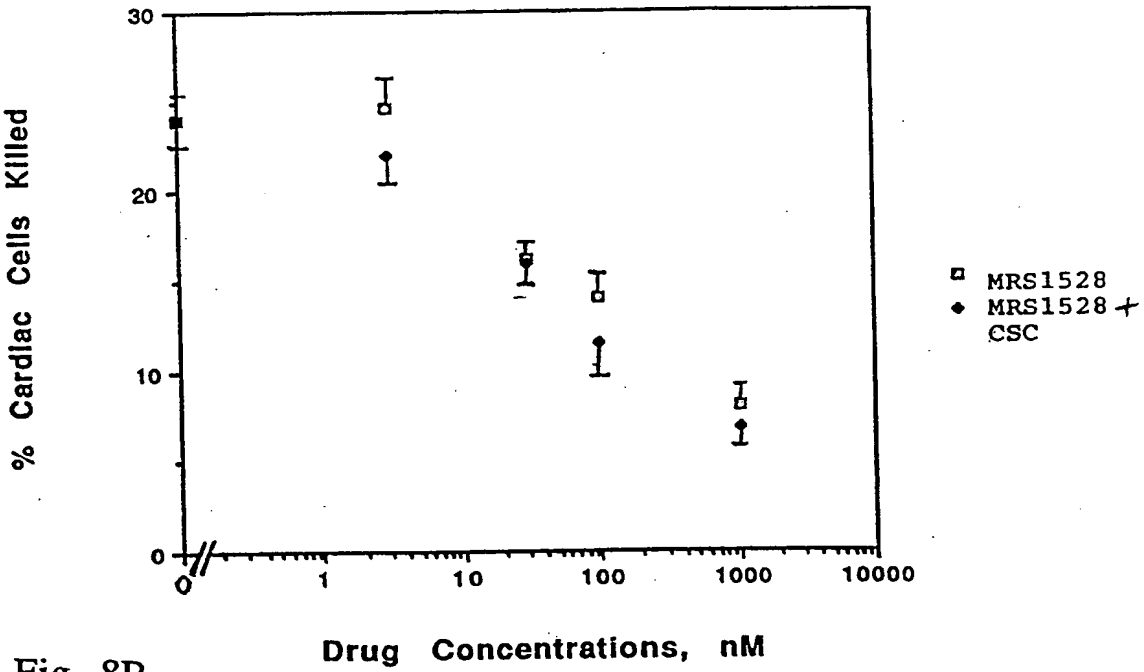
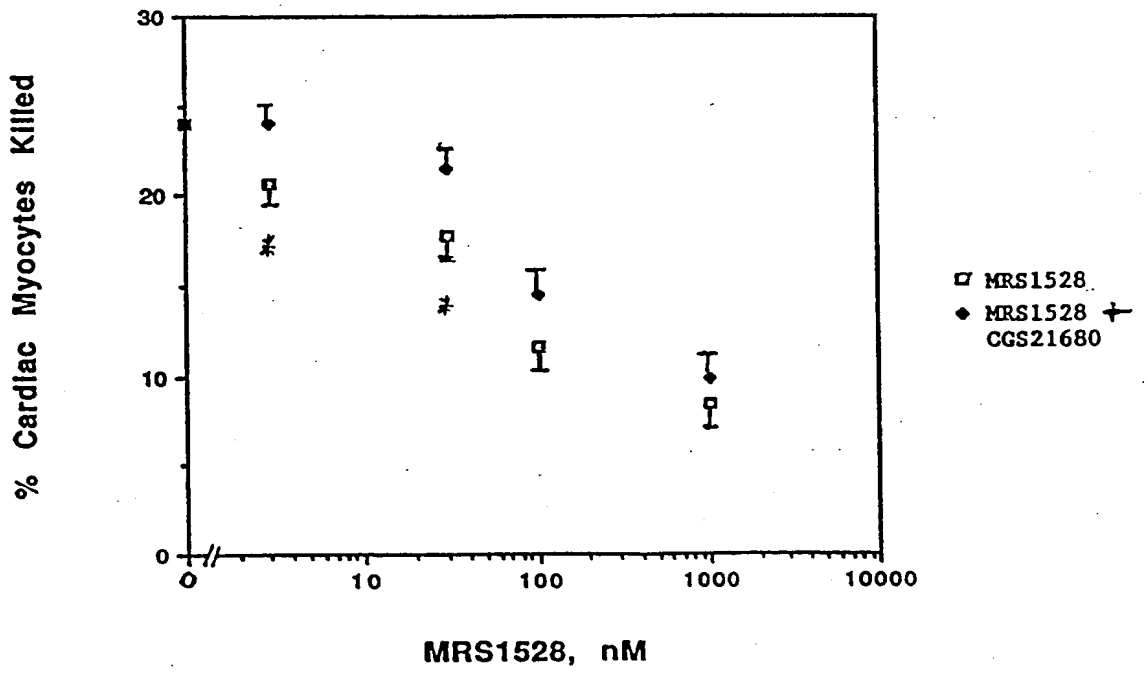


Fig. 8B



* significantly different from those determined in the presence of CGS21680

Fig. 8C

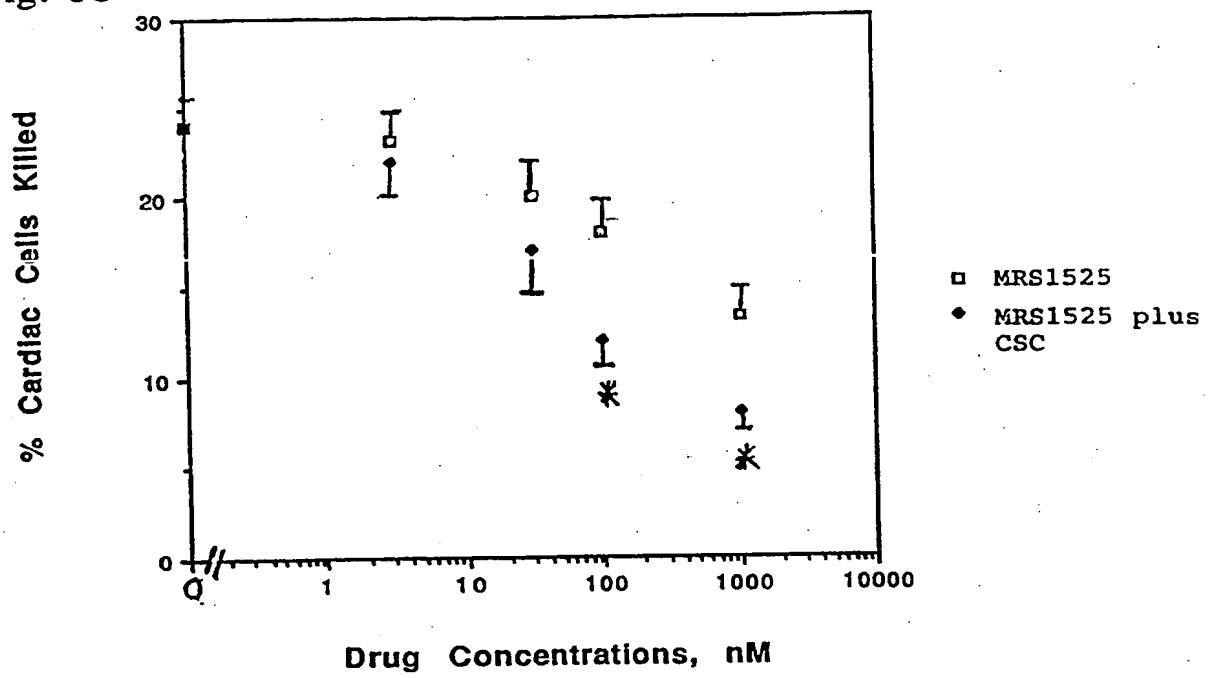

% Cardiac Cells Killed
30
20
10
0
0
1
10
100
1000
10000
Drug Concentrations, nM
MRS1525
MRS1525 plus CSC

Fig. 8D

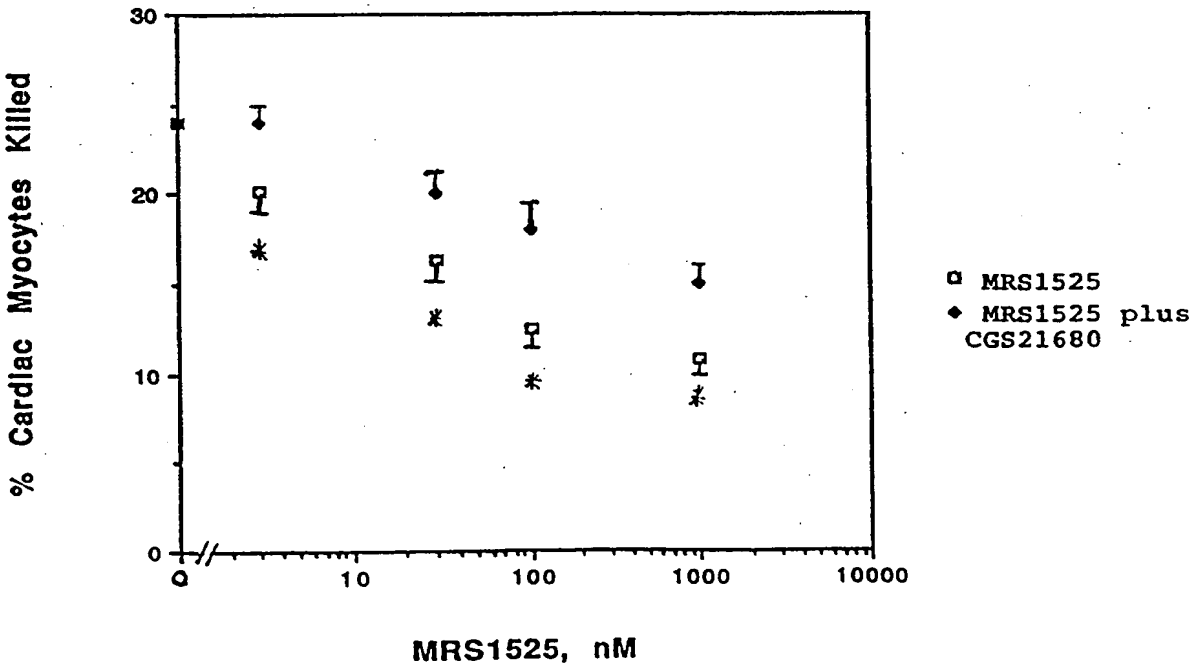
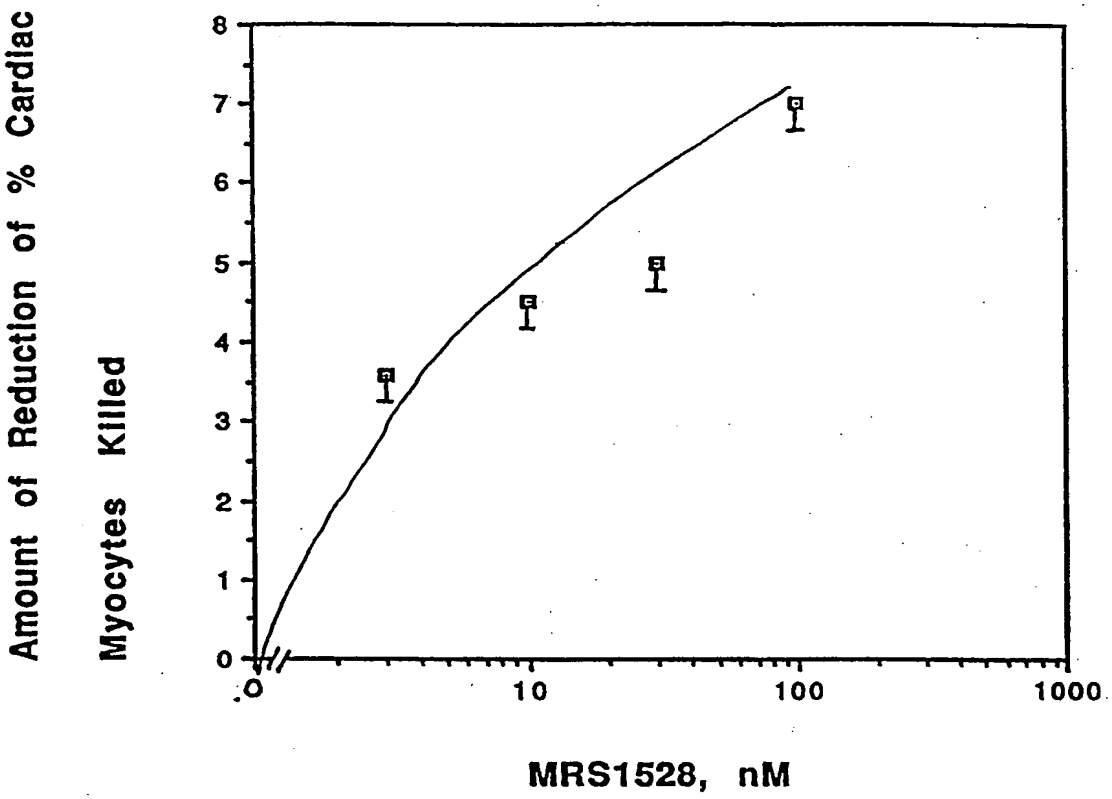

% Cardiac Myocytes Killed
30
20
10
0
0
10
100
1000
10000
MRS1525, nM
MRS1525
MRS1525 plus CGS21680

Fig. 8E



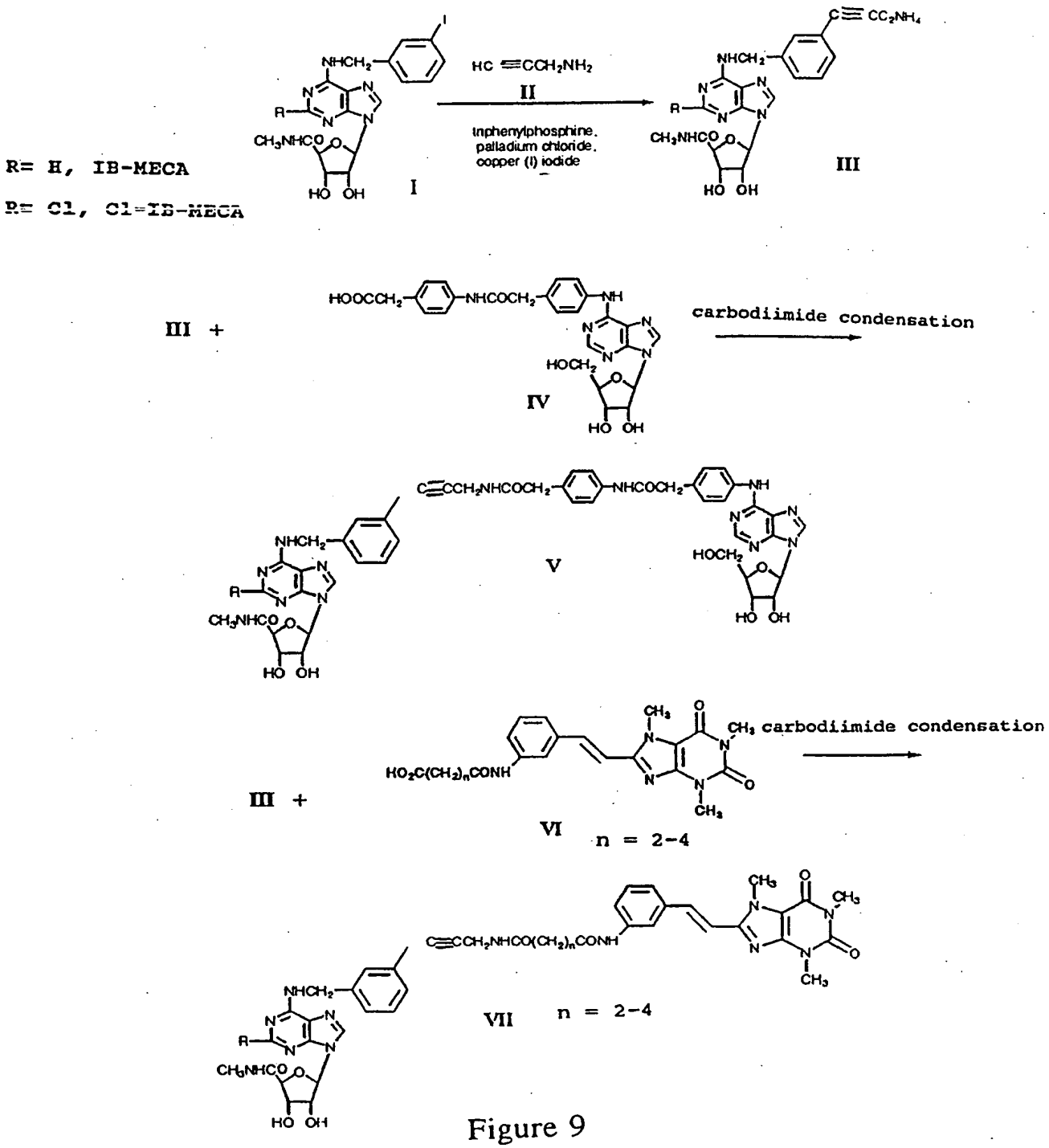
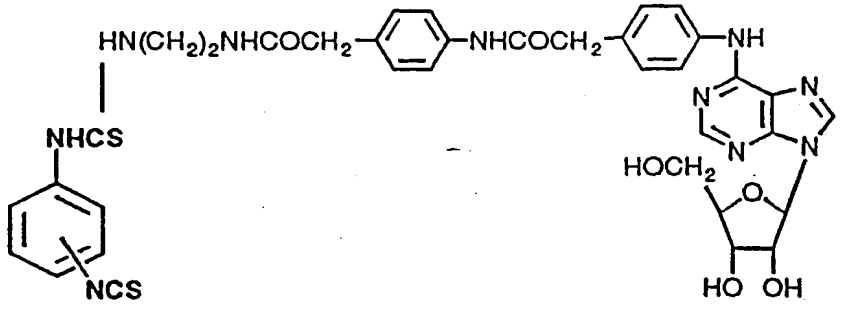

R= H, IB-MECA
R= Cl, Cl-IB-MECA
HC ≡CCH2NH2
II
triphenylphosphine, palladium chloride, copper (I) iodide
I
III
III +
IV
carbodiimide condensation
V
III +
VI
n = 2-4
carbodiimide condensation
VII
n = 2-4

Figure 9

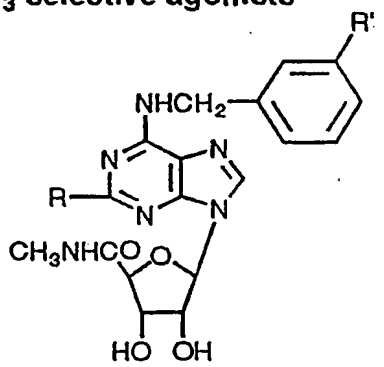
Fig. 10A

Derivatization of A_1 selective agonist for coupling to amine-derivatized A_3 agonist



m- and p-DITC-ADAC

Derivatization of A_3 selective agonists



R' = C—CCH_2NHR''; R = H, Cl

for coupling to isothiocaynates or carboxylic acids:

R'' = H, $COCH(R''')NH_2$ [D- or L- amino acid],

for coupling to amines:

R'' =$CSNHC_6H_4$-NCS (p- or m-),

$COCH(R''')NHCSNHC_6H_4$-NCS (p- or m-)

as A_3 agonists for exploring SAR:

R'' =$COCH_3$, $COCH(R''')NHCOOC(CH_3)_3$ [Boc-D- or L- amino acid],

R''' = any naturally occurring amino acid

Fig. 10B

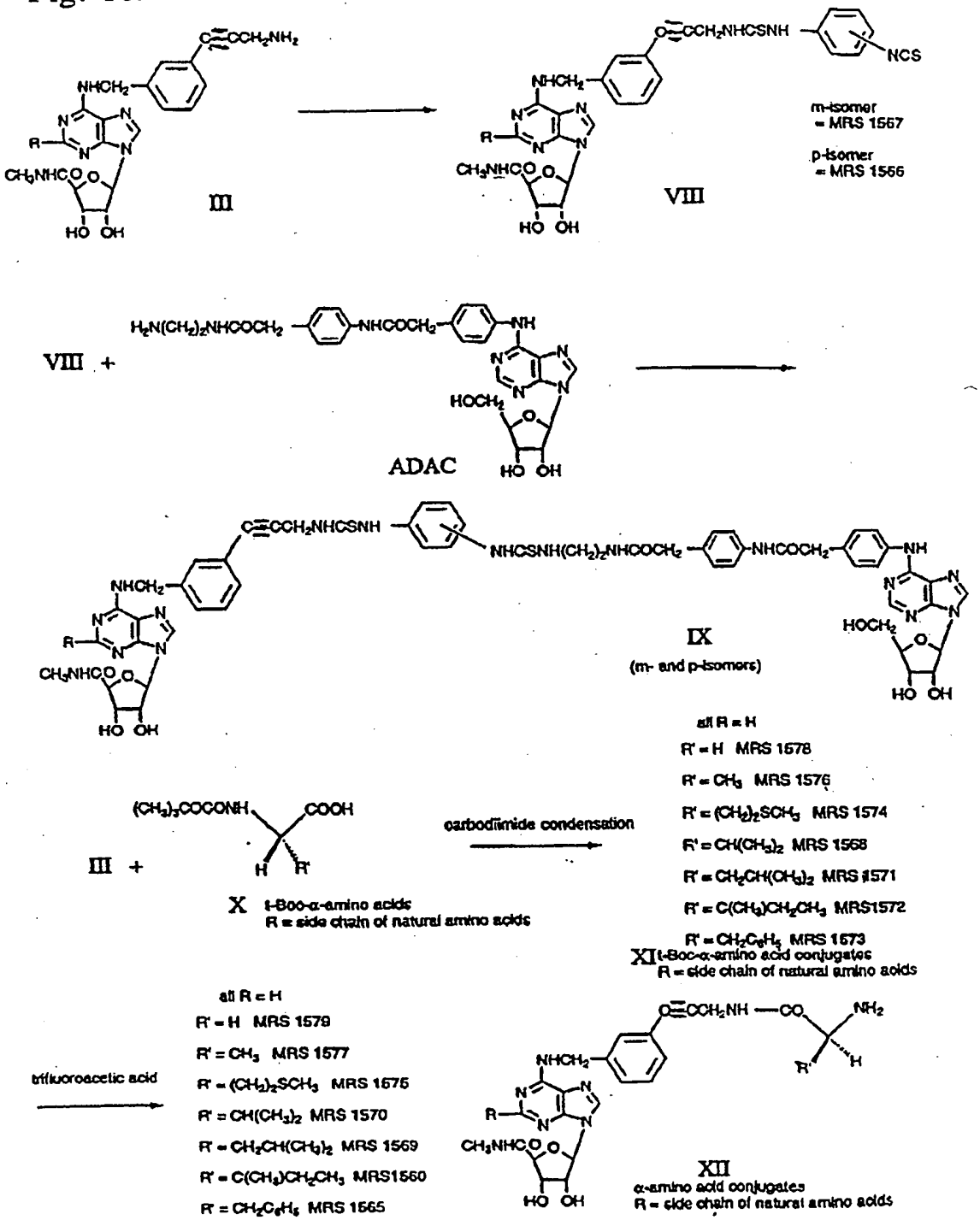
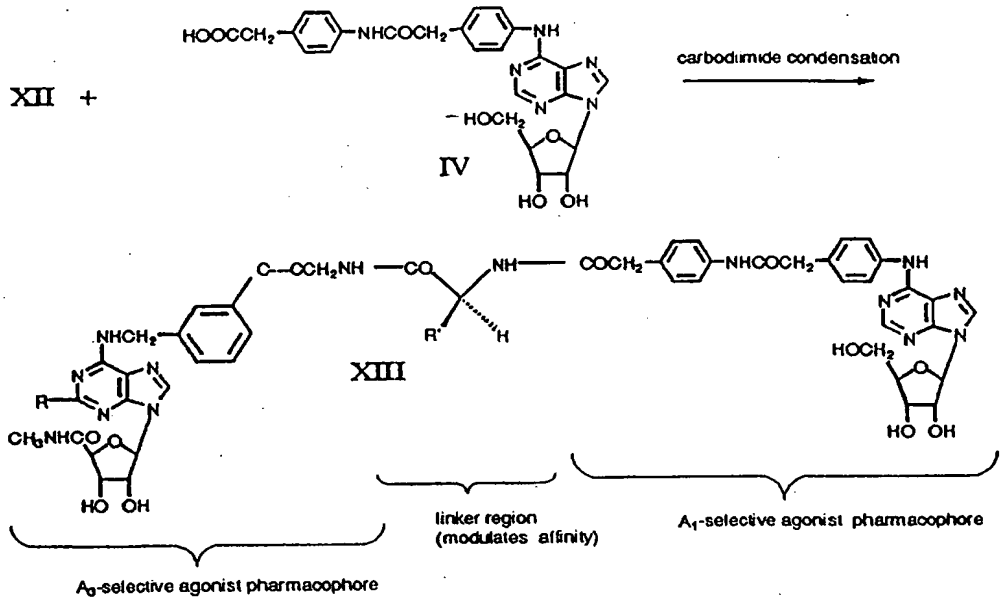

III
VIII
m-isomer = MRS 1567
p-isomer = MRS 1566
VIII +
ADAC
IX
(m- and p-isomers)
all R = H
R' = H MRS 1578
R' = CH3 MRS 1576
R' = (CH2)2SCH3 MRS 1574
R' = CH(CH3)2 MRS 1568
R' = CH2CH(CH3)2 MRS 1571
R' = C(CH3)CH2CH3 MRS1572
R' = CH2C6H5 MRS 1573
XI t-Boc-α-amino acid conjugates
R = side chain of natural amino acids
III +
carbodiimide condensation
X t-Boc-α-amino acids
R = side chain of natural amino acids
trifluoroacetic acid
all R = H
R' = H MRS 1579
R' = CH3 MRS 1577
R' = (CH2)2SCH3 MRS 1575
R' = CH(CH3)2 MRS 1570
R' = CH2CH(CH3)2 MRS 1569
R' = C(CH3)CH2CH3 MRS1560
R' = CH2C6H5 MRS 1565
XII
α-amino acid conjugates
R = side chain of natural amino acids

Fig. 10C

Synthesis of binary conjugate with extended linker



Synthesis of binary conjugate with short linker

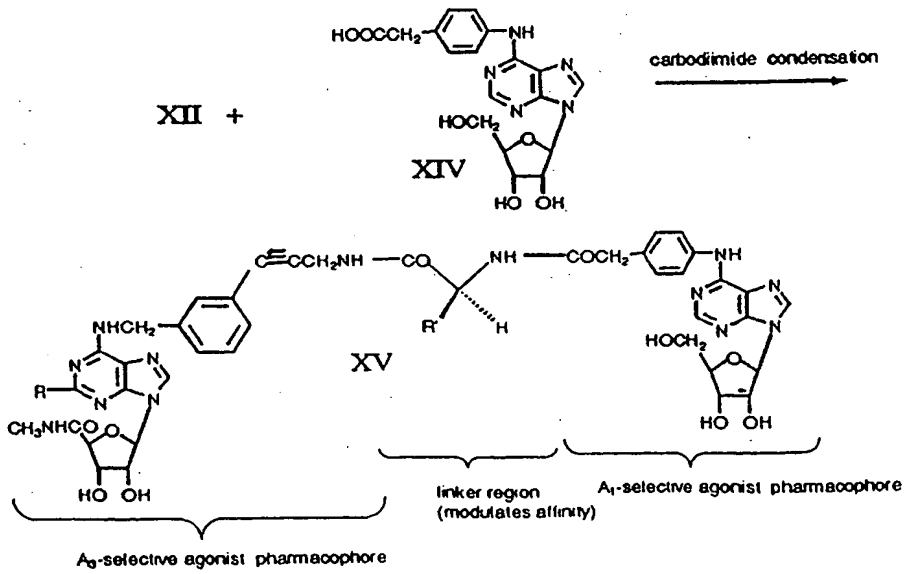


Fig. 11A

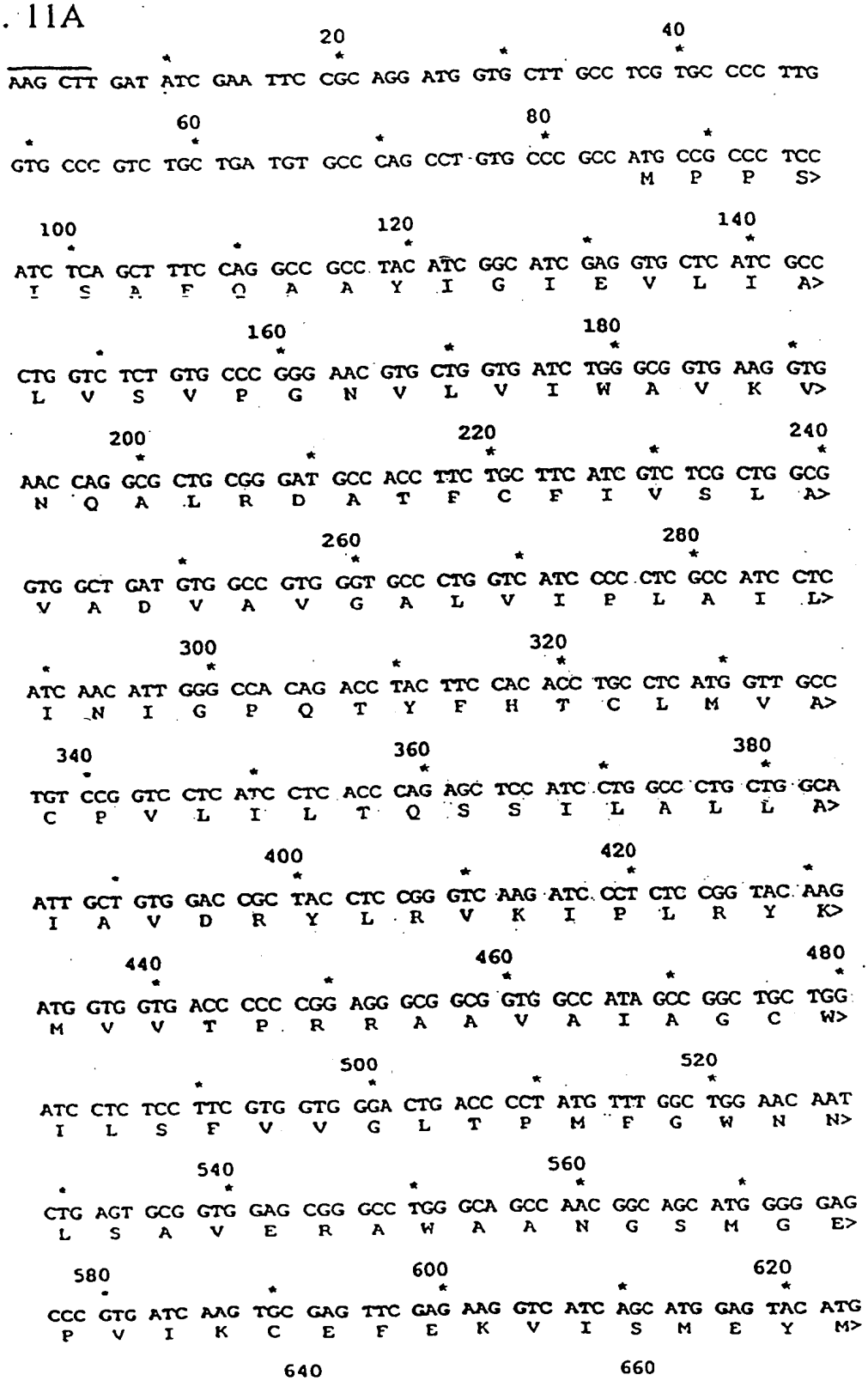

20
40
AAG CTT GAT ATC GAA TTC CGC AGG ATG GTG CTT GCC TCG TGC CCC TTG
60
80
GTG CCC GTC TGC TGA TGT GCC CAG CCT GTG CCC GCC ATG CCG CCC TCC
M P P S>
100
120
140
ATC TCA GCT TTC CAG GCC GCC TAC ATC GGC ATC GAG GTG CTC ATC GCC
I S A F Q A A Y I G I E V L I A>
160
180
CTG GTC TCT GTG CCC GGG AAC GTG CTG GTG ATC TGG GCG GTG AAG GTG
L V S V P G N V L V I W A V K V>
200
220
240
AAC CAG GCG CTG CGG GAT GCC ACC TTC TGC TTC ATC GTC TCG CTG GCG
N Q A L R D A T F C F I V S L A>
260
280
GTG GCT GAT GTG GCC GTG GGT GCC CTG GTC ATC CCC CTC GCC ATC CTC
V A D V A V G A L V I P L A I L>
300
320
ATC AAC ATT GGG CCA CAG ACC TAC TTC CAC ACC TGC CTC ATG GTT GCC
I N I G P Q T Y F H T C L M V A>
340
360
380
TGT CCG GTC CTC ATC CTC ACC CAG AGC TCC ATC CTG GCC CTG CTG GCA
C P V L I L T Q S S I L A L L A>
400
420
ATT GCT GTG GAC CGC TAC CTC CGG GTC AAG ATC CCT CTC CGG TAC AAG
I A V D R Y L R V K I P L R Y K>
440
460
480
ATG GTG GTG ACC CCC CGG AGG GCG GCG GTG GCC ATA GCC GGC TGC TGG
M V V T P R R A A V A I A G C W>
500
520
ATC CTC TCC TTC GTG GTG GGA CTG ACC CCT ATG TTT GGC TGG AAC AAT
I L S F V V G L T P M F G W N N>
540
560
CTG AGT GCG GTG GAG CGG GCC TGG GCA GCC AAC GGC AGC ATG GGG GAG
L S A V E R A W A A N G S M G E>
580
600
620
CCC GTG ATC AAG TGC GAG TTC GAG AAG GTC ATC AGC ATG GAG TAC ATG
P V I K C E F E K V I S M E Y M>
640
660

Fig. 11B

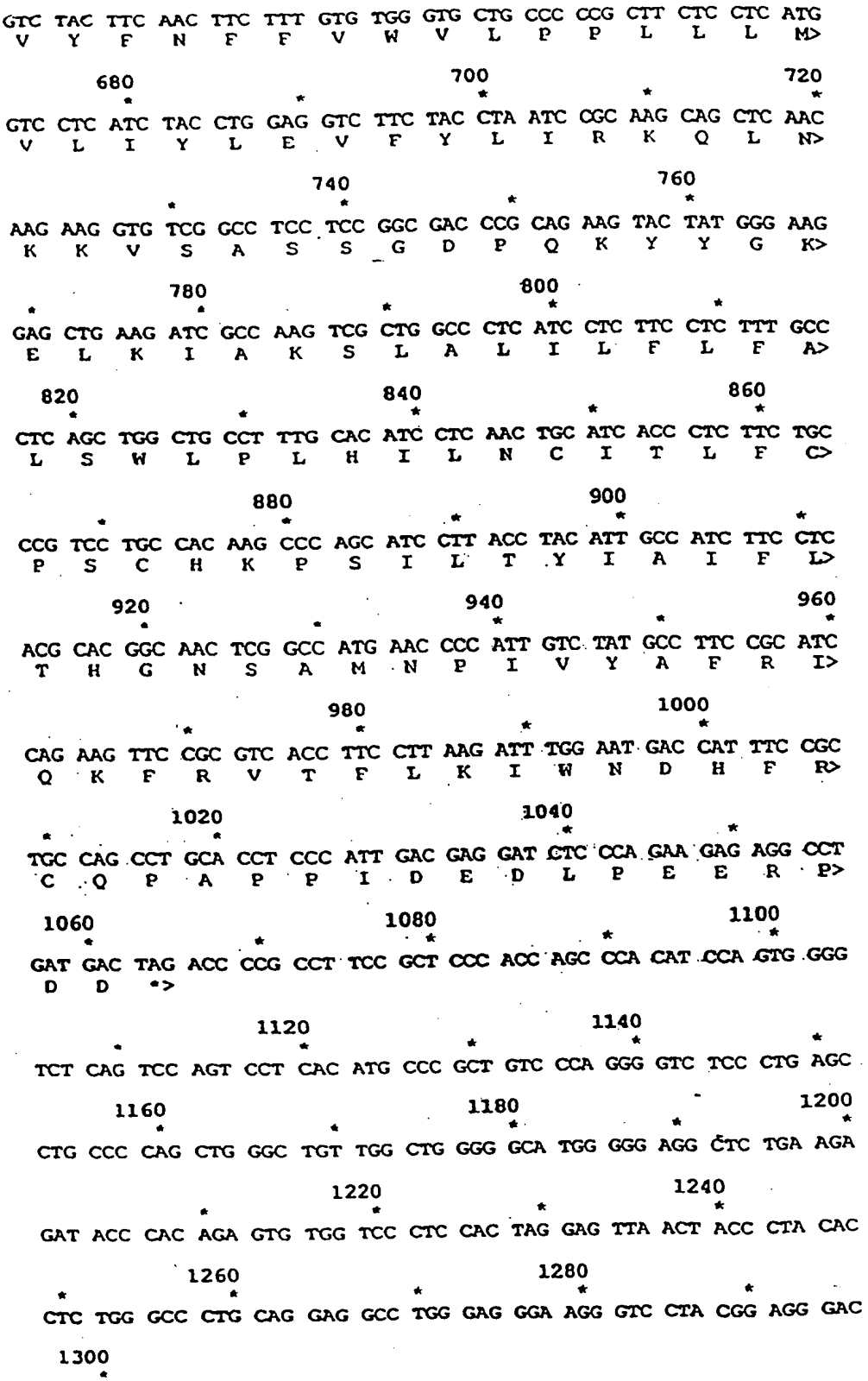

GTC TAC TTC AAC TTC TTT GTG TGG GTG CTG CCC CCG CTT CTC CTC ATG
V Y F N F F V W V L P P L L L M>
680
700
720
GTC CTC ATC TAC CTG GAG GTC TTC TAC CTA ATC CGC AAG CAG CTC AAC
V L I Y L E V F Y L I R K Q L N>
740
760
AAG AAG GTG TCG GCC TCC TCC GGC GAC CCG CAG AAG TAC TAT GGG AAG
K K V S A S S G D P Q K Y Y G K>
780
800
GAG CTG AAG ATC GCC AAG TCG CTG GCC CTC ATC CTC TTC CTC TTT GCC
E L K I A K S L A L I L F L F A>
820
840
860
CTC AGC TGG CTG CCT TTG CAC ATC CTC AAC TGC ATC ACC CTC TTC TGC
L S W L P L H I L N C I T L F C>
880
900
CCG TCC TGC CAC AAG CCC AGC ATC CTT ACC TAC ATT GCC ATC TTC CTC
P S C H K P S I L T Y I A I F L>
920
940
960
ACG CAC GGC AAC TCG GCC ATG AAC CCC ATT GTC TAT GCC TTC CGC ATC
T H G N S A M N P I V Y A F R I>
980
1000
CAG AAG TTC CGC GTC ACC TTC CTT AAG ATT TGG AAT GAC CAT TTC CGC
Q K F R V T F L K I W N D H F R>
1020
1040
TGC CAG CCT GCA CCT CCC ATT GAC GAG GAT CTC CCA GAA GAG AGG CCT
C Q P A P P I D E D L P E E R P>
1060
1080
1100
GAT GAC TAG ACC CCG CCT TCC GCT CCC ACC AGC CCA CAT CCA GTG GGG
D D *>
1120
1140
TCT CAG TCC AGT CCT CAC ATG CCC GCT GTC CCA GGG GTC TCC CTG AGC
1160
1180
1200
CTG CCC CAG CTG GGC TGT TGG CTG GGG GCA TGG GGG AGG CTC TGA AGA
1220
1240
GAT ACC CAC AGA GTG TGG TCC CTC CAC TAG GAG TTA ACT ACC CTA CAC
1260
1280
CTC TGG GCC CTG CAG GAG GCC TGG GAG GGA AGG GTC CTA CGG AGG GAC
1300

Fig. 12A

```
          10          20          30          40
-CAA TTT TCA GCT GTT CTT TGC TCA ATA ATA ACT TTT TTA TCA CCA AGA

 50          60          70          80          90
TAT CTC TCT AAG TTT TTG ACA TAT TCC TCA TTT GTT TTG ATA AAA GTT

  100         110         120         130         140
TTC TTA TTT TCT TAG AAA AAT AAG TTA CTA AAA GTC ATA TAT CAT TGT

    150         160         170         180         190
ATA TCT TCA AAA TAT TGC TTA AAA CTA GGA CTT GTA TTT AAA TGT TTT

       200         210         220         230         240
TTC TTC TTA AAG ACA ATT TGC AGG TGC CCT CAG GAA CCC TGA AGC TGG

          250         260         270         280
GCT GAG CCA TGA TGC TGC TGC CAG AAC CCC TGC AGA GGG CCT GGT TTC

290         300         310         320         330
AGG AGA CTC AGA GTC CTC TGT GAA AAA GCC CTT GGA GAG CGC CCC AGC

  340         350         360         370         380
AGG GCT GCA CTT GGC TCC TGT GAG GAA GGG GCT CAG GGG TCT GGG CCC

    390         400         410         420         430
CTC CGC CTG GGC CGG GCT GGG AGC CAG GCG GGC GGC TGG GCT GCA GCA

       440         450         460         470         480
AAT GGA CCG TGA GCT GGC CCA GCC CGC GTC CGT GCT GAG CCT GCC TGT

          490         500         510         520         530
CGT CTG TGG CC ATG CCC ATC ATG GGC TCC TCG GTG TAC ATC ACG GTG GAG
               M   P   I   M   G   S   S   V   Y   I   T   V   E>

          540         550         560         570
CTG GCC ATT GCT GTG CTG GCC ATC CTG GGC AAT GTG CTG GTG TGC TGG
 L   A   I   A   V   L   A   I   L   G   N   V   L   V   C   W>

580         590         600         610         620
GCC GTG TGG CTC AAC AGC AAC CTG CAG AAC GTC ACC AAC TAC TTT GTG
 A   V   W   L   N   S   N   L   Q   N   V   T   N   Y   F   V>

  630         640         650         660         670
GTG TCA CTG GCG GCG GCC GAC ATC GCA GTG GGT GTG CTC GCC ATC CCC
 V   S   L   A   A   A   D   I   A   V   G   V   L   A   I   P>

    680         690         700         710         720
TTT GCC ATC ACC ATC AGC ACC GGG TTC TGC GCT GCC TGC CAC GGC TGC
```

Fig. 12B

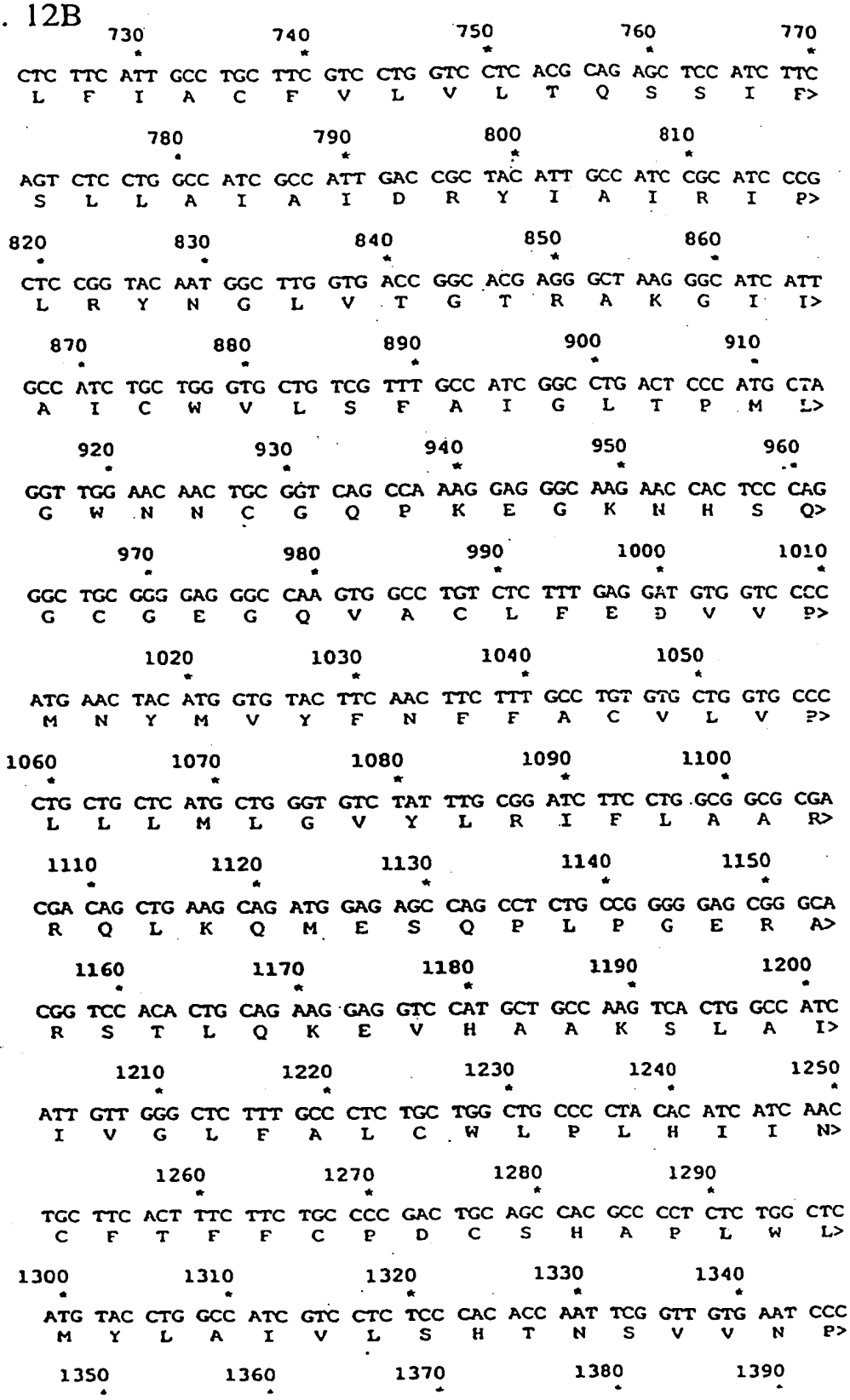

730 740 750 760 770
CTC TTC ATT GCC TGC TTC GTC CTG GTC CTC ACG CAG AGC TCC ATC TTC
L F I A C F V L V L T Q S S I F>
780 790 800 810
AGT CTC CTG GCC ATC GCC ATT GAC CGC TAC ATT GCC ATC CGC ATC CCG
S L L A I A I D R Y I A I R I P>
820 830 840 850 860
CTC CGG TAC AAT GGC TTG GTG ACC GGC ACG AGG GCT AAG GGC ATC ATT
L R Y N G L V T G T R A K G I I>
870 880 890 900 910
GCC ATC TGC TGG GTG CTG TCG TTT GCC ATC GGC CTG ACT CCC ATG CTA
A I C W V L S F A I G L T P M L>
920 930 940 950 960
GGT TGG AAC AAC TGC GGT CAG CCA AAG GAG GGC AAG AAC CAC TCC CAG
G W N N C G Q P K E G K N H S Q>
970 980 990 1000 1010
GGC TGC GGG GAG GGC CAA GTG GCC TGT CTC TTT GAG GAT GTG GTC CCC
G C G E G Q V A C L F E D V V P>
1020 1030 1040 1050
ATG AAC TAC ATG GTG TAC TTC AAC TTC TTT GCC TGT GTG CTG GTG CCC
M N Y M V Y F N F F A C V L V P>
1060 1070 1080 1090 1100
CTG CTG CTC ATG CTG GGT GTC TAT TTG CGG ATC TTC CTG GCG GCG CGA
L L L M L G V Y L R I F L A A R>
1110 1120 1130 1140 1150
CGA CAG CTG AAG CAG ATG GAG AGC CAG CCT CTG CCG GGG GAG CGG GCA
R Q L K Q M E S Q P L P G E R A>
1160 1170 1180 1190 1200
CGG TCC ACA CTG CAG AAG GAG GTC CAT GCT GCC AAG TCA CTG GCC ATC
R S T L Q K E V H A A K S L A I>
1210 1220 1230 1240 1250
ATT GTT GGG CTC TTT GCC CTC TGC TGG CTG CCC CTA CAC ATC ATC AAC
I V G L F A L C W L P L H I I N>
1260 1270 1280 1290
TGC TTC ACT TTC TTC TGC CCC GAC TGC AGC CAC GCC CCT CTC TGG CTC
C F T F F C P D C S H A P L W L>
1300 1310 1320 1330 1340
ATG TAC CTG GCC ATC GTC CTC TCC CAC ACC AAT TCG GTT GTG AAT CCC
M Y L A I V L S H T N S V V N P>
1350 1360 1370 1380 1390

Fig. 12C

```
TTC ATC TAC GCC TAC CGT ATC CGC GAG TTC CGC CAG ACC TTC CGC AAG
 F   I   Y   A   Y   R   I   R   E   F   R   Q   T   F   R   K>

   1400        1410        1420        1430        1440
    *           *           *           *           *
ATC ATT CGC AGC CAC GTC CTG AGG CAG CAA GAA CCT TTC AAG GCA GCT
 I   I   R   S   H   V   L   R   Q   Q   E   P   F   K   A   A>

      1450        1460        1470        1480        1490
       *           *           *           *           *
GGC ACC AGT GCC CGG GTC TTG GCA GCT CAT GGC AGT GTC GGA GAG CAG
 G   T   S   A   R   V   L   A   A   H   G   S   V   G   E   Q>

         1500        1510        1520        1530
          *           *           *           *
GTC AGC CTC CGT CTC AAC GGC CAC CCG CCA GAG GTG TGG GCC AAC GGC
 V   S   L   R   L   N   G   H   P   P   E   V   W   A   N   G>

1540        1550        1560        1570        1580
 *           *           *           *           *
AGT GCT CCC CAC CCT GAG CGG AGG CCC AAT GGC TAC GCC CTG GGG CTG
 S   A   P   H   P   E   R   R   P   N   G   Y   A   L   G   L>

  1590        1600        1610        1620        1630
   *           *           *           *           *
GTG AGT GGA GGG AGT GCC CAA GAG TCC CAG GGG AAC ACG GGC CTC CCA
 V   S   G   G   S   A   Q   E   S   Q   G   N   T   G   L   P>

    1640        1650        1660        1670        1680
     *           *           *           *           *
GAC GTG GAG CTC CTT AGC CAT GAG CTC AAG AGA GTG TGC CCA GAG CCC
 D   V   E   L   L   S   H   E   L   K   R   V   C   P   E   P>

      1690        1700        1710        1720        1730
       *           *           *           *           *
CCT GGC CTA GAT GAC CCC CTG GCC CAG GAT GGA GCA GGA GTG TCC TGA T
 P   G   L   D   D   P   L   A   Q   D   G   A   G   V   S   *>

        1740        1750        1760        1770
         *           *           *           *
GAT TCA TGG AGT TTG CCC CTT CCT AAG GGA AGG AGA TCT TTA TCT TTC

1780        1790        1800        1810        1820
 *           *           *           *           *
TGG TTG GCT TGA CCA GTC ACG TTG GGA GAA GAG AGA GAG TGC CAG GAG

  1830        1840        1850        1860        1870
   *           *           *           *           *
ACC CTG AGG GCA GCC GGT TCC TAC TTT GGA CTG AGA GAA GGG AGC CCC

    1880        1890        1900        1910        1920
     *           *           *           *           *
AGG CTG GAG CAG CAT GAG GCC CAG CAA GAA GGG CTT GGG TTC TGA GGA

      1930        1940        1950        1960        1970
       *           *           *           *           *
AGC AGA TGT TTC ATG CTG TGA GGC CTT GCA CCA GGT GGG GGC CAC AGC

        1980        1990        2000        2010
         *           *           *           *
ACC AGC AGC ATC TTT GCT GGG CAG GGC CCA GCC CTC CAC TGC AGA AGC

2020        2030        2040        2050        2060
 *           *           *           *           *
ATC TGG AAG CAC CAC CTT GTC TCC ACA GAG CAG CTT GGG CAC AGC AGA

2070        2080        2090        2100        2110
```

Fig. 12D

```
                                                                    *
CTG GCC TGG CCC TGA GAC TGG GGA GTG GCT CCA ACA GCC TCC TGC CAC

  2120          2130          2140          2150          2160
   *             *             *             *             *
CCA CAC ACC ACT CTC CCT AGA CTC TCC TAG GGT TCA GGA GCT GCT GGG

       2170          2180          2190          2200          2210
        *             *             *             *             *
CCC AGA GGT GAC ATT TGA CTT TTT TTC CAG GAA AAA TGT AAG TGT GAG

         2220          2230          2240          2250
          *             *             *             *
GAA ACC CTT TTT ATT TTA TTA CCT TTC ACT CTC TGG CTG CTG GGT CTG

2260          2270          2280          2290          2300
 *             *             *             *             *
CCG TCG GTC CTG CTG CTA ACC TGG CAC CAG AGC CTC TGC CCG GGG AGC

 2310          2320          2330          2340          2350
  *             *             *             *             *
CTC AGG CAG TCC TCT CCT GCT GTC ACA GCT GCC ATC CAC TTC TCA GTC

   2360          2370          2380          2390          2400
    *             *             *             *             *
CCA GGG CCA TCT CTT GGA GTG ACA AAG CTG GGA TCA AGG ACA GGG AGT

       2410          2420          2430          2440          2450
        *             *             *             *             *
TGT AAC AGA GCA GTG CCA GAG CAT GGG CCC AGG TCC CAG GGG AGA GGT

         2460          2470          2480          2490
          *             *             *             *
TGG GGC TGG CAG GCC ACT GGC ATG TGC TGA GTA GCG CAG AGC TAC CCA

2500          2510          2520          2530          2540
 *             *             *             *             *
GTG AGA GGC CTT GTC TAA CTG CCT TTC CTT CTA AAG GGA ATG TTT TTT

 2550          2560          2570
  *             *             *
TCT GAG ATA AAA TAA AAA CGA GCC ACA G
```

Fig. 13A

```
          10          20          30          40          50          60
          *           *           *           *           *           *
-GGACCTCTGGGAAGACGTCTGGCGAGAGCTAGGCCCACTGGCCCTACAGACGGATCTTGC

          70          80          90         100         110
          *           *           *           *           *
TGGCTCACCTGTCCCTGTGGAGGTTCCCCTGGGAAGGCAAG ATG CCC AAC AAC
                                          Met Pro Asn Asn>

       120         130         140         150
        *           *           *           *
AGC ACT GCT CTG TCA TTG GCC AAT GTT ACC TAC ATC ACC ATG GAA
Ser Thr Ala Leu Ser Leu Ala Asn Val Thr Tyr Ile Thr Met Glu>

160         170         180         190         200
 *           *           *           *           *
ATT TTC ATT GGA CTC TGC GCC ATA GTG GGC AAC GTG CTG GTC ATC
Ile Phe Ile Gly Leu Cys Ala Ile Val Gly Asn Val Leu Val Ile>

       210         220         230         240
        *           *           *           *
TGC GTG GTC AAG CTG AAC CCC AGC CTG CAG ACC ACC ACC TTC TAT
Cys Val Val Lys Leu Asn Pro Ser Leu Gln Thr Thr Thr Phe Tyr>

250         260         270         280         290
 *           *           *           *           *
TTC ATT GTC TCT CTA GCC CTG GCT GAC ATT GCT GTT GGG GTG CTG
Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala Val Gly Val Leu>

       300         310         320         330
        *           *           *           *
GTC ATG CCT TTG GCC ATT GTT GTC AGC CTG GGC ATC ACA ATC CAC
Val Met Pro Leu Ala Ile Val Val Ser Leu Gly Ile Thr Ile His>

340         350         360         370         380
 *           *           *           *           *
TTC TAC AGC TGC CTT TTT ATG ACT TGC CTA CTG CTT ATC TTT ACC
Phe Tyr Ser Cys Leu Phe Met Thr Cys Leu Leu Leu Ile Phe Thr>

       390         400         410         420
        *           *           *           *
CAC GCC TCC ATC ATG TCC TTG CTG GCC ATC GCT GTG GAC CGA TAC
His Ala Ser Ile Met Ser Leu Leu Ala Ile Ala Val Asp Arg Tyr>

430         440         450         460         470
 *           *           *           *           *
TTG CGG GTC AAG CTT ACC GTC AGA TAC AAG AGG GTC ACC ACT CAC
Leu Arg Val Lys Leu Thr Val Arg Tyr Lys Arg Val Thr Thr His>

       480         490         500         510
        *           *           *           *
AGA AGA ATA TGG CTG GCC CTG GGC CTT TGC TGG CTG GTG TCA TTC
Arg Arg Ile Trp Leu Ala Leu Gly Leu Cys Trp Leu Val Ser Phe>

520         530         540         550         560
 *           *           *           *           *
CTG GTG GGA TTG ACC CCC ATG TTT GGC TGG AAC ATG AAA CTG ACC
Leu Val Gly Leu Thr Pro Met Phe Gly Trp Asn Met Lys Leu Thr>

       570         580         590         600
```

Fig. 13B

```
                *           *           *           *
TCA GAG TAC CAC AGA AAT GTC ACC TTC CTT TCA TGC CAA TTT GTT
Ser Glu Tyr His Arg Asn Val Thr Phe Leu Ser Cys Gln Phe Val>

610         620         630         640         650
 *           *           *           *           *
TCC GTC ATG AGA ATG GAC TAC ATG GTA TAC TTC AGC TTC CTC ACC
Ser Val Met Arg Met Asp Tyr Met Val Tyr Phe Ser Phe Leu Thr>

      660         670         680         690
        *           *           *           *
TGG ATT TTC ATC CCC CTG GTT GTC ATG TGC GCC ATC TAT CTT GAC
Trp Ile Phe Ile Pro Leu Val Val Met Cys Ala Ile Tyr Leu Asp>

700         710         720         730         740
 *           *           *           *           *
ATC TTT TAC ATC ATT CGG AAC AAA CTC AGT CTG AAC TTA TCT AAC
Ile Phe Tyr Ile Ile Arg Asn Lys Leu Ser Leu Asn Leu Ser Asn>

      750         760         770         780
        *           *           *           *
TCC AAA GAG ACA GGT GCA TTT TAT GGA CGG GAG TTC AAG ACG GCT
Ser Lys Glu Thr Gly Ala Phe Tyr Gly Arg Glu Phe Lys Thr Ala>

790         800         810         820         830
 *           *           *           *           *
AAG TCC TTG TTT CTG GTT CTT TTC TTG TTT GCT CTG TCA TGG CTG
Lys Ser Leu Phe Leu Val Leu Phe Leu Phe Ala Leu Ser Trp Leu>

      840         850         860         870
        *           *           *           *
CCT TTA TCT ATC ATC AAC TGC ATC ATC TAC TTT AAT GGT GAG GTA
Pro Leu Ser Ile Ile Asn Cys Ile Ile Tyr Phe Asn Gly Glu Val>

880         890         900         910         920
 *           *           *           *           *
CCA CAG CTT GTG CTG TAC ATG GGC ATC CTG CTG TCC CAT GCC AAC
Pro Gln Leu Val Leu Tyr Met Gly Ile Leu Leu Ser His Ala Asn>

      930         940         950         960
        *           *           *           *
TCC ATG ATG AAC CCT ATC GTC TAT GCC TAT AAA ATA AAG AAG TTC
Ser Met Met Asn Pro Ile Val Tyr Ala Tyr Lys Ile Lys Lys Phe>

970         980         990         1000        1010
 *           *           *           *           *
AAG GAA ACC TAC CTT TTG ATC CTC AAA GCC TGT GTG GTC TGC CAT
Lys Glu Thr Tyr Leu Leu Ile Leu Lys Ala Cys Val Val Cys His>

      1020        1030        1040        1050
        *           *           *           *
CCC TCT GAT TCT TTG GAC ACA AGC ATT GAG AAG AAT TCT GAG TAG
Pro Ser Asp Ser Leu Asp Thr Ser Ile Glu Lys Asn Ser Glu ***>

1060      1070      1080      1090      1100      1110
 *         *         *         *         *         *
TTATCCATCAGAGATGACTCTGTCTCATTGACCTTCAGATTCCCCATCAACAAACACTTG

1120      1130      1140      1150      1160      1170
 *         *         *         *         *         *
AGGGCCTGTATGCCTGGGCCAAGGGATTTTTACATCCTTGATTACTTCCACTGAGGTGGG
```

Fig. 13 C

```
1180       1190       1200       1210       1220       1230
  *          *          *          *          *          *
AGCATCTCCAGTGCTCCCCAATTATATCTCCCCCACTCCACTACTCTCTTCCTCCACTTC

1240       1250       1260       1270       1280       1290
  *          *          *          *          *          *
ATTTTTCCTTTGTCCTTTCTCTCTAATTCAGTGTTTTGGAGGCCTGACTTGGGGACAACG

1300       1310       1320       1330       1340       1350
  *          *          *          *          *          *
TATTATTGATATTATTGTCTGTTTTCCTTCTTCCCAATAGAAGAATAAGTCATGGAGCCT

1360       1370
  *          *
GAAGGGTGCCTAGTTGAC
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09031

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70
US CL : 514/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/46

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, DERWENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,657,897 A (BRISTOL et al.) 14 April 1987, see entire document.	1-63

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 SEPTEMBER 1998	Date of mailing of the international search report 20 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ZOHREH FAY Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

Fig. 1A

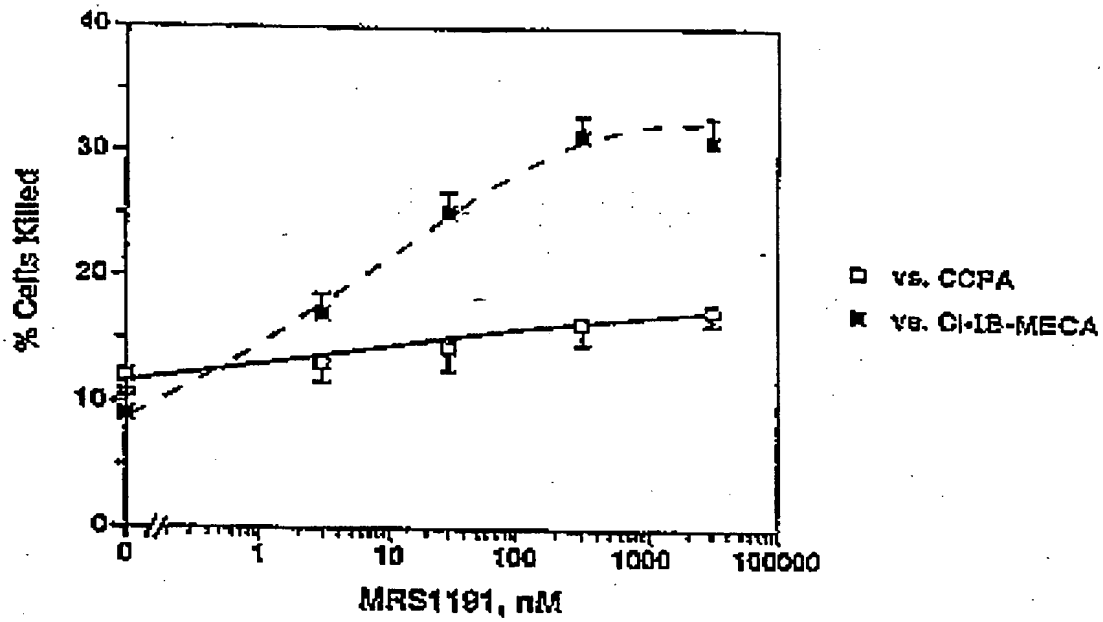
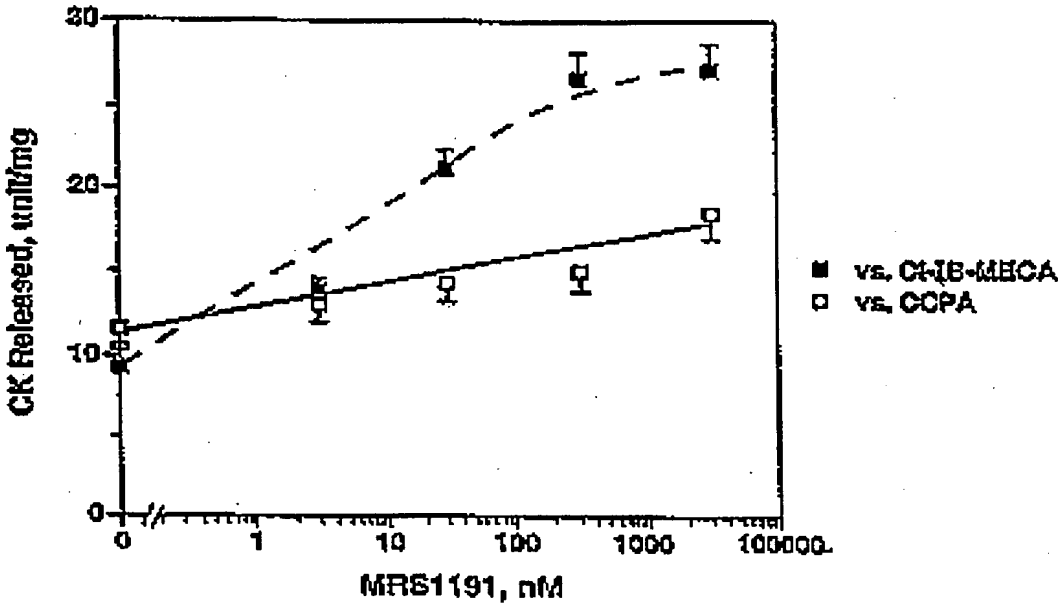


Fig. 1B



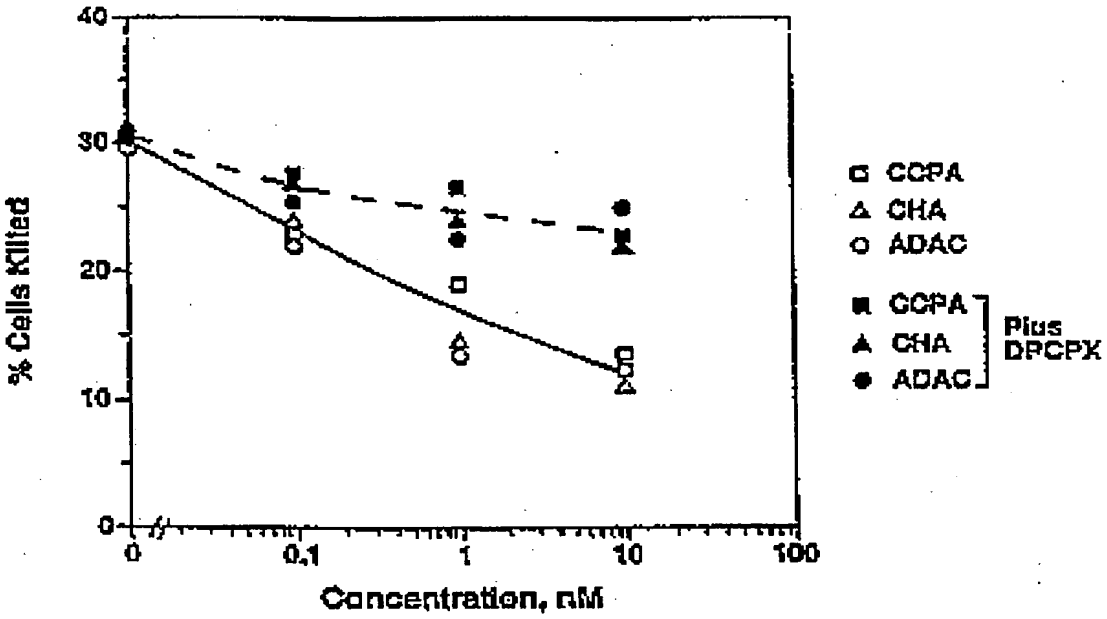

40
30
20
10
0
% Cells Killed
0
0.1
1
10
100
Concentration, nM
CCPA
CHA
ADAC
CCPA
CHA
ADAC
Plus DPCPX

Figure 2

Fig. 3A

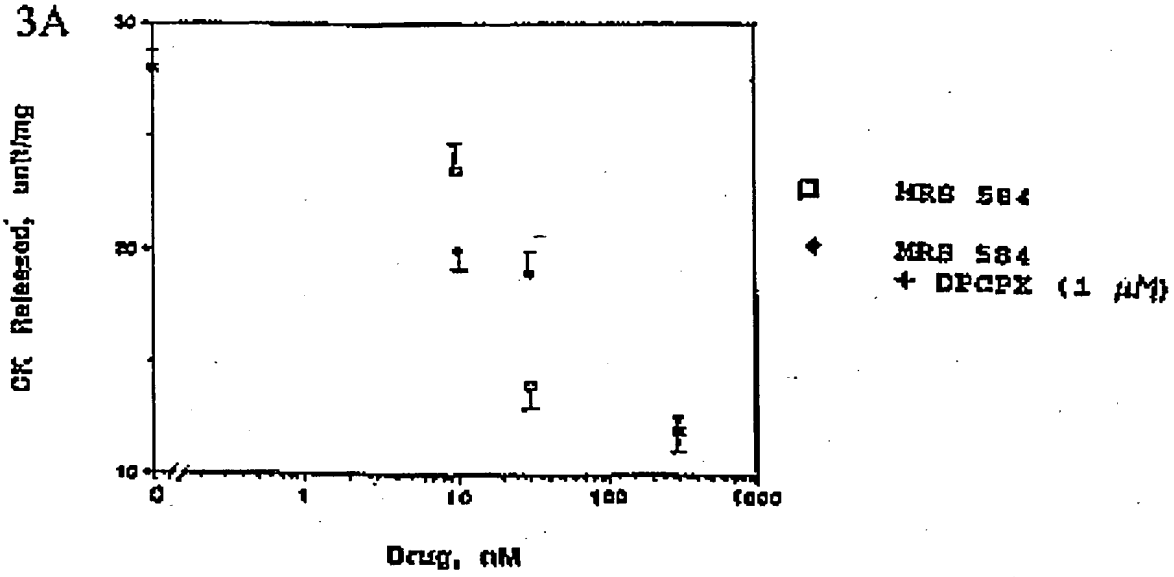


Fig. 3B

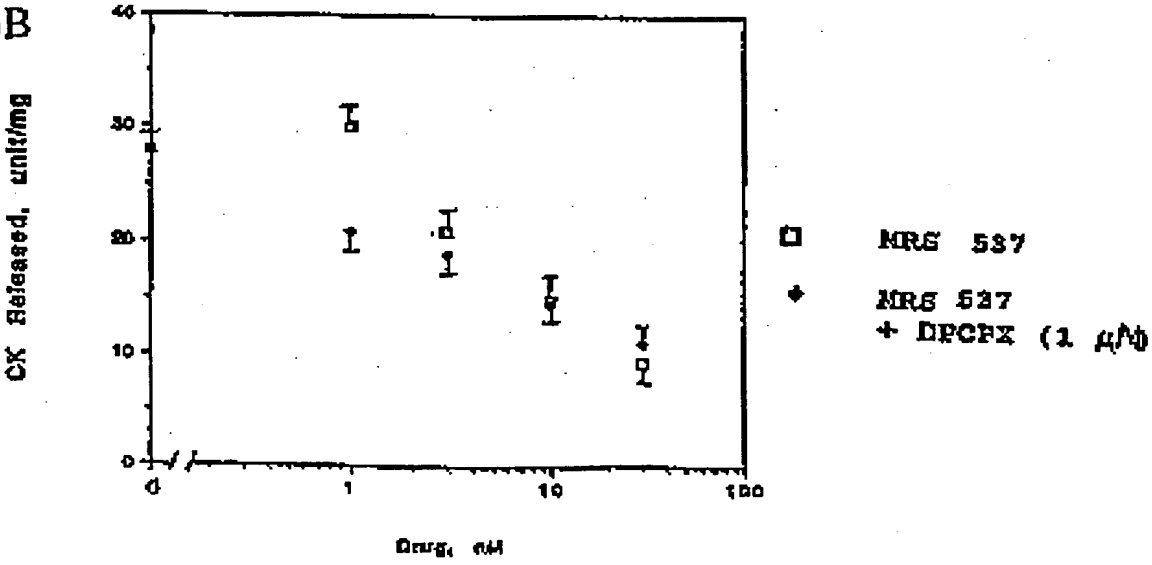


Fig. 3C

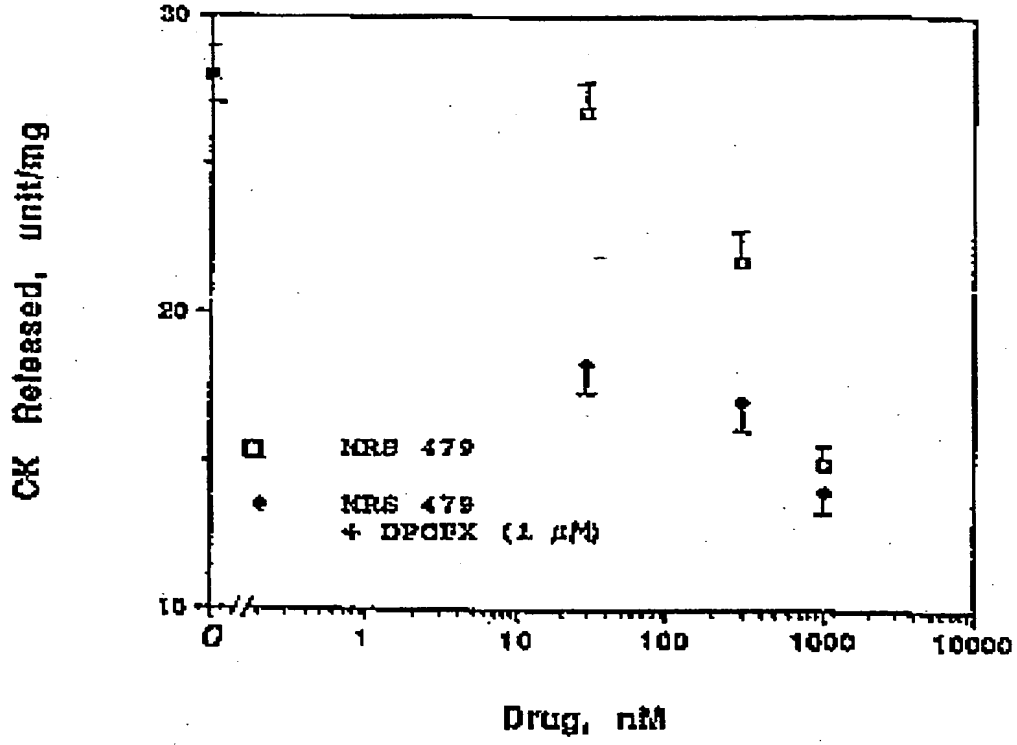

CK Released, unit/mg
30
20
10
0
1
10
100
1000
10000
MRS 479
MRS 479
+ DPCPX (1 μM)
Drug, nM

Fig. 3D

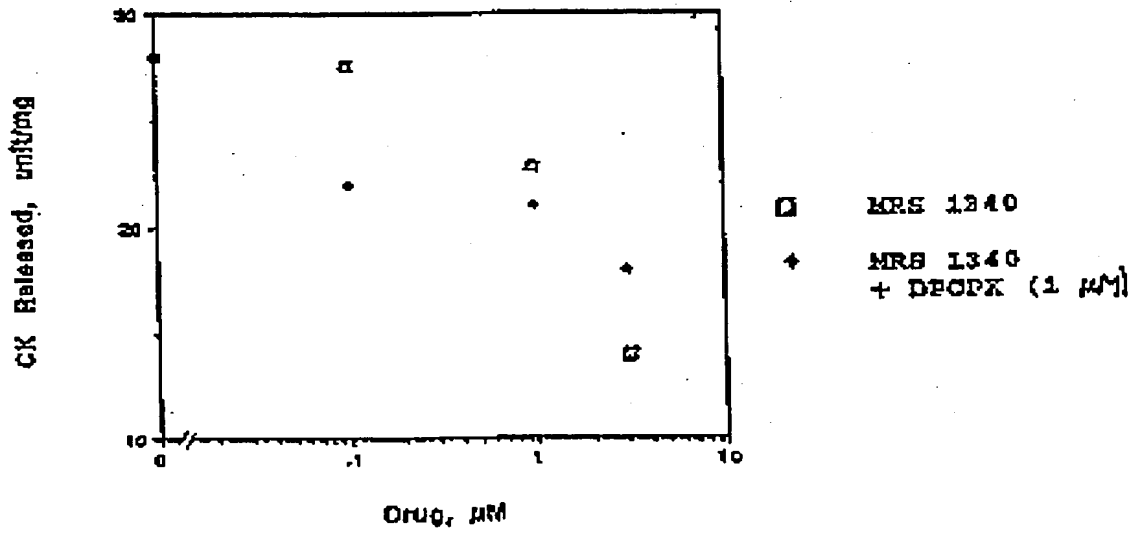

CK Released, unit/mg
0
.1
1
10
MRS 1340
MRS 1340
+ DPCPX (1 μM)
Drug, μM

Fig. 3E

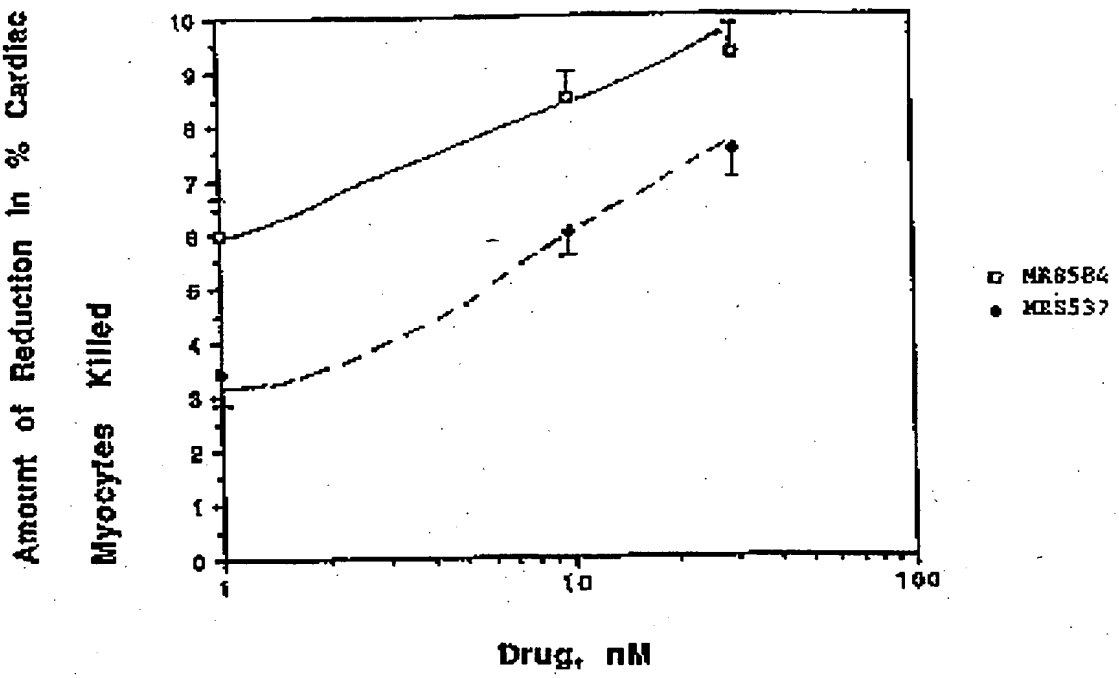


Fig. 3F

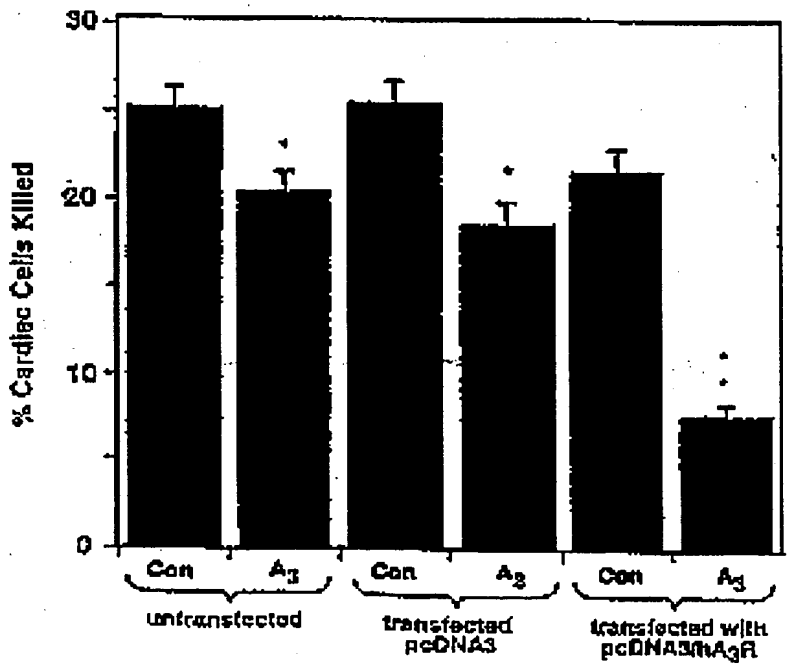


Fig. 4A

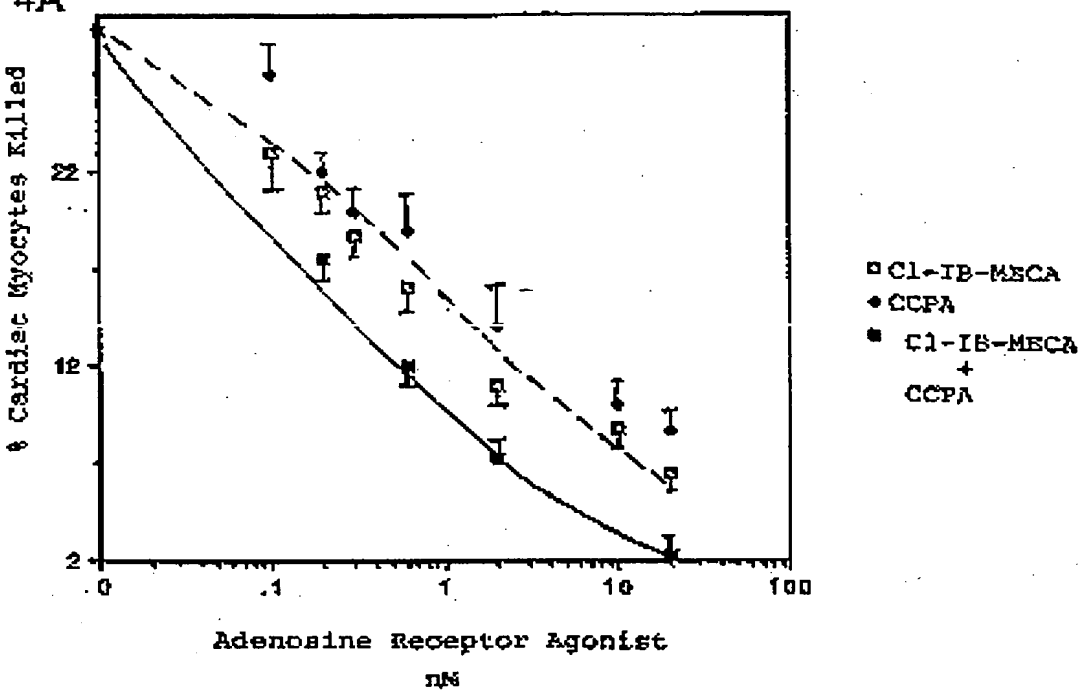

% Cardiac Myocytes Killed
22
12
2
.0
.1
1
10
100
Adenosine Receptor Agonist
nM
Cl-IB-MECA
CCPA
Cl-IB-MECA + CCPA

Fig. 4B

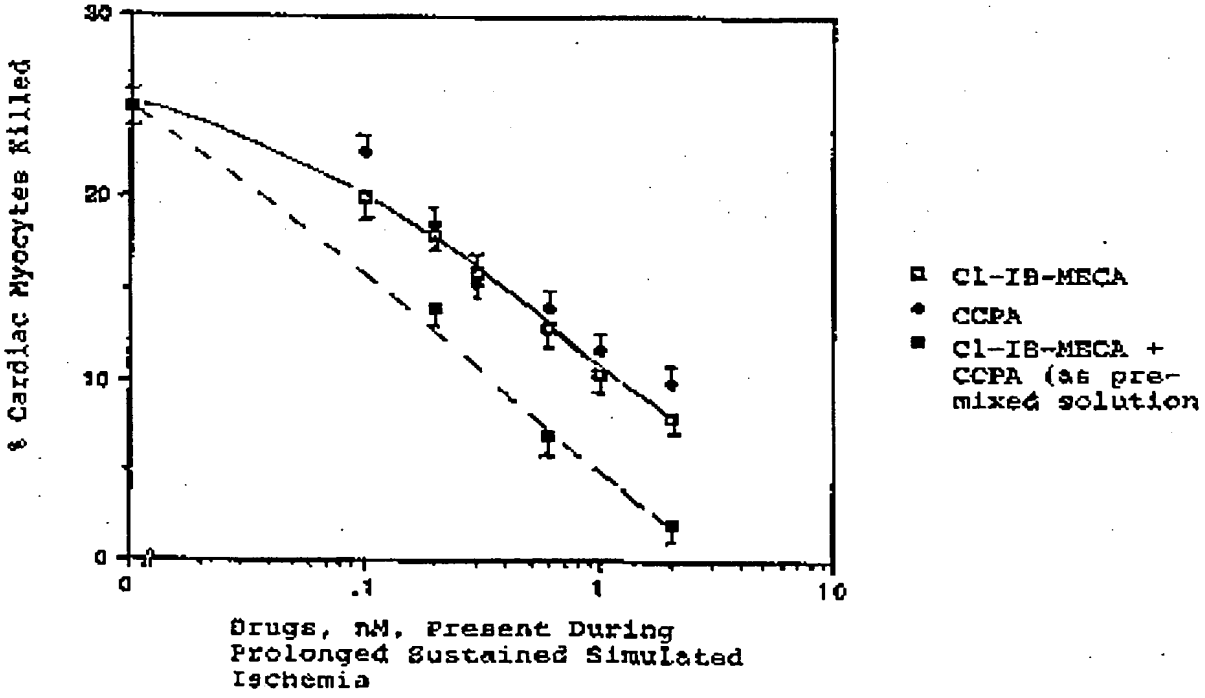

% Cardiac Myocytes Killed
30
20
10
0
0
.1
1
10
Drugs, nM, Present During Prolonged Sustained Simulated Ischemia
Cl-IB-MECA
CCPA
Cl-IB-MECA + CCPA (as pre-mixed solution

Fig. 5A

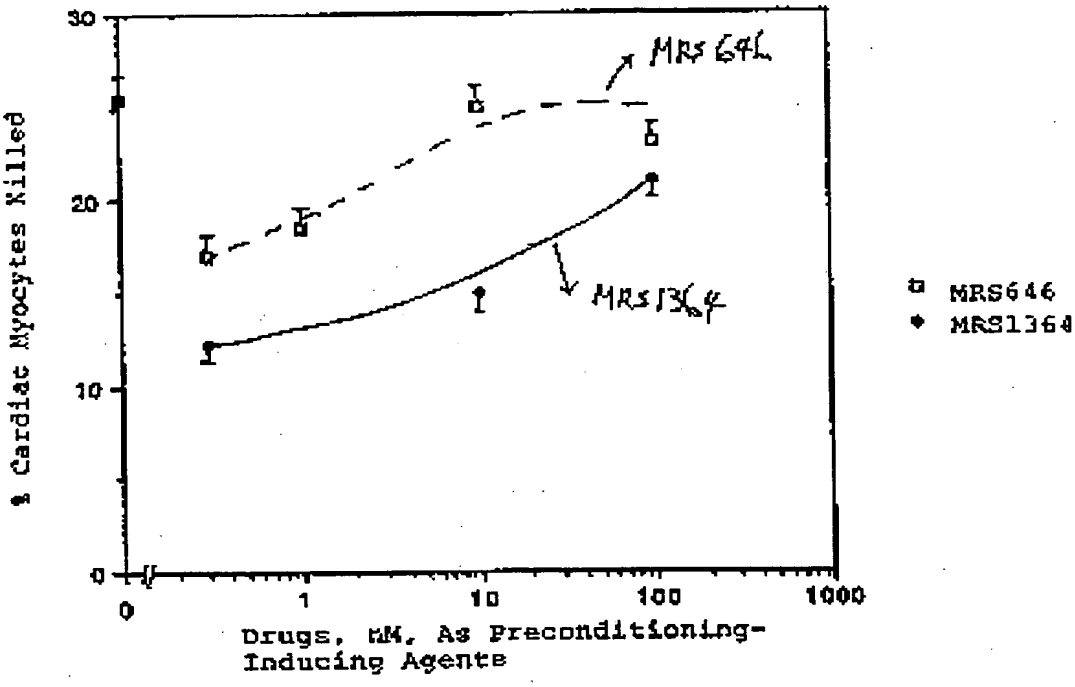

30
20
10
0
% Cardiac Myocytes Killed
MRS 646
MRS1364
0
1
10
100
1000
Drugs, nM, As Preconditioning-Inducing Agents
MRS646
MRS1364

Fig. 5B

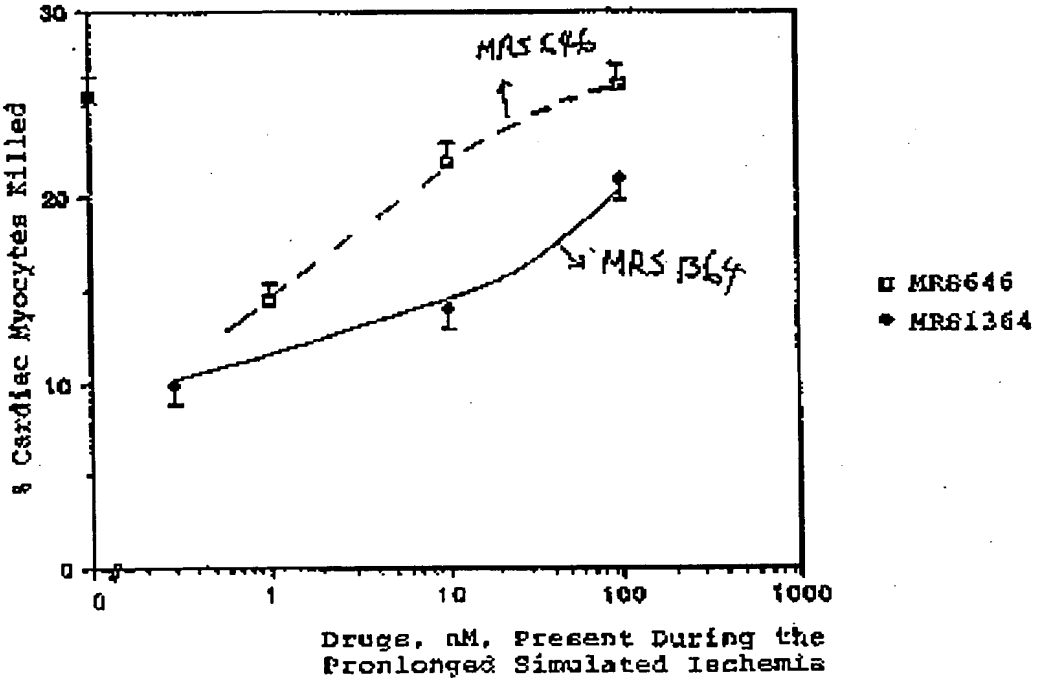

30
20
10
0
% Cardiac Myocytes Killed
MRS 646
MRS 1364
0
1
10
100
1000
Druge, nM, Present During the Pronlonged Simulated Ischemia
MR6646
MR61364

Fig. 5C

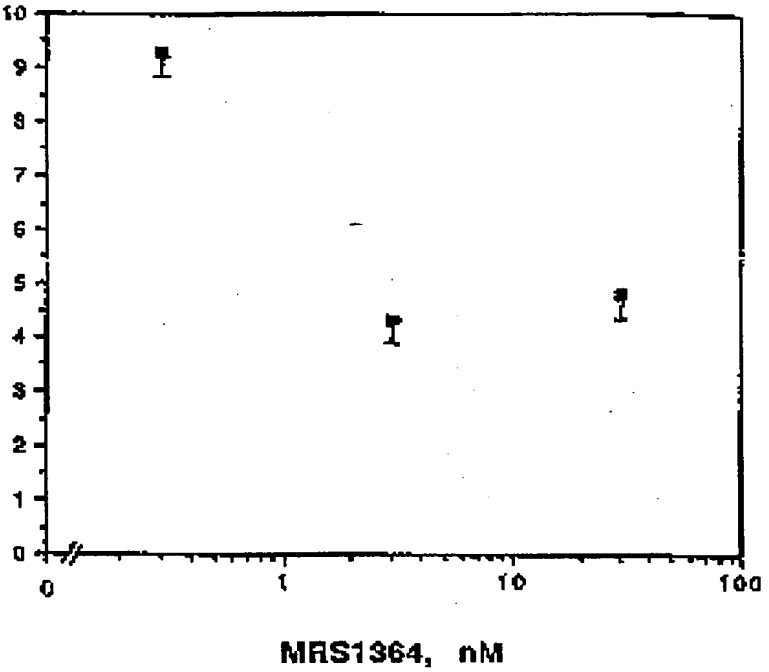

% Cardiac Myocytes Killed
0
1
2
3
4
5
6
7
8
9
10
0
1
10
100
MRS1364, nM

Fig. 5D

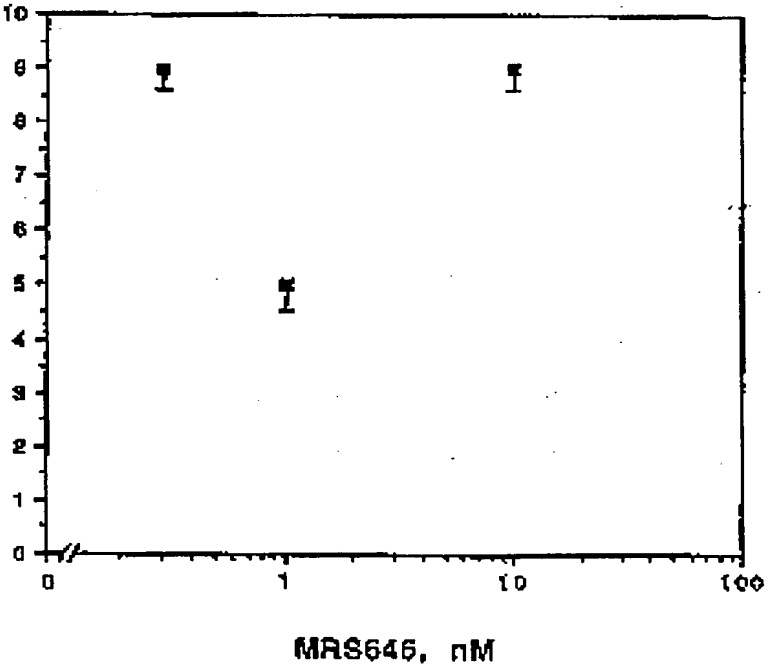

% Cardiac Myocytes Killed
0
1
2
3
4
5
6
7
8
9
10
0
1
10
100
MRS646, nM

Fig. 6A

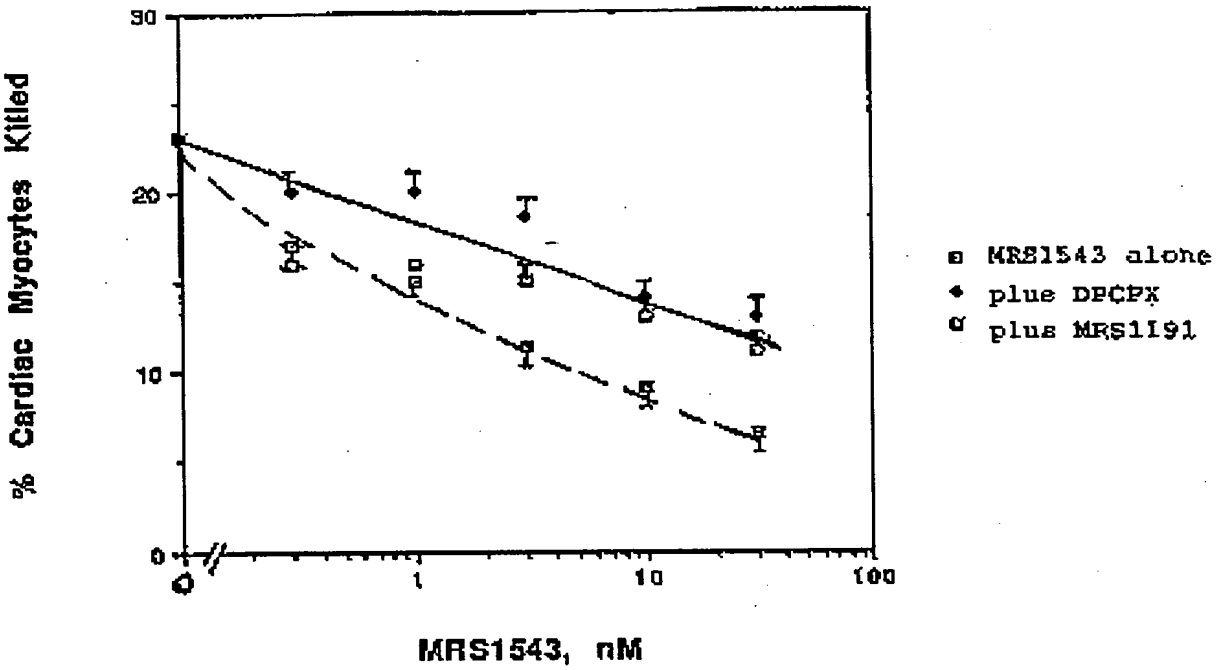

% Cardiac Myocytes Killed
30
20
10
0
0
1
10
100
MRS1543, nM
MRS1543 alone
plue DPCPX
plus MRS1191

Fig. 6B

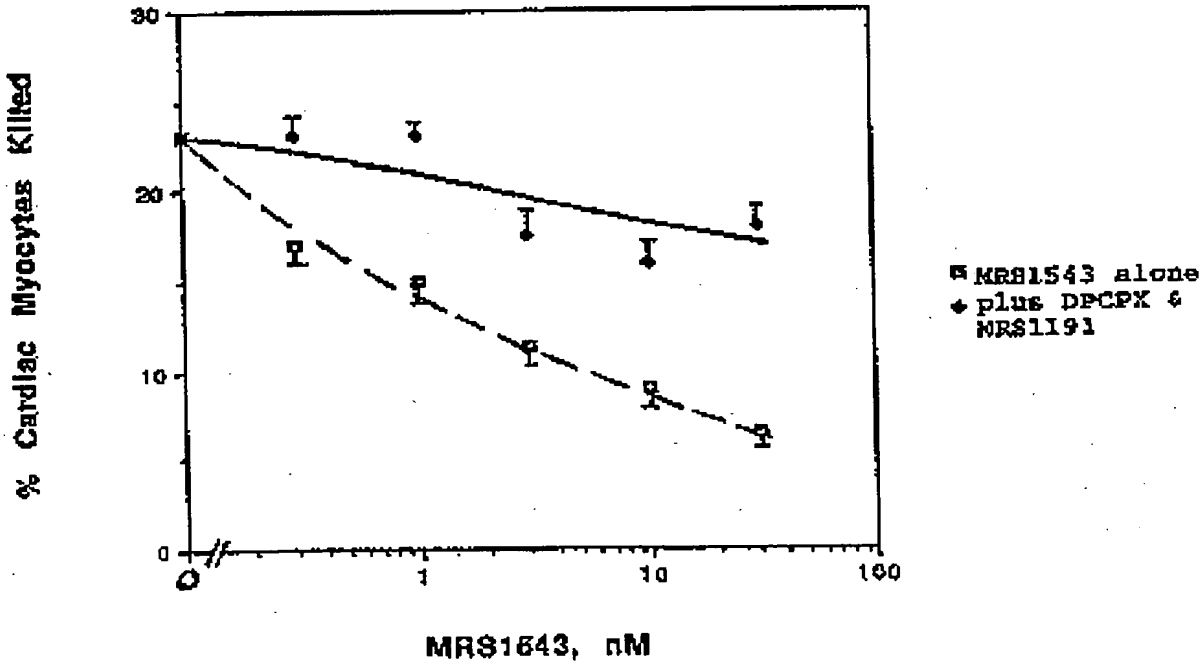

% Cardiac Myocytes Killed
30
20
10
0
0
1
10
100
MRS1643, nM
MRS1543 alone
plus DPCPX &
MRS1191

Fig. 6C

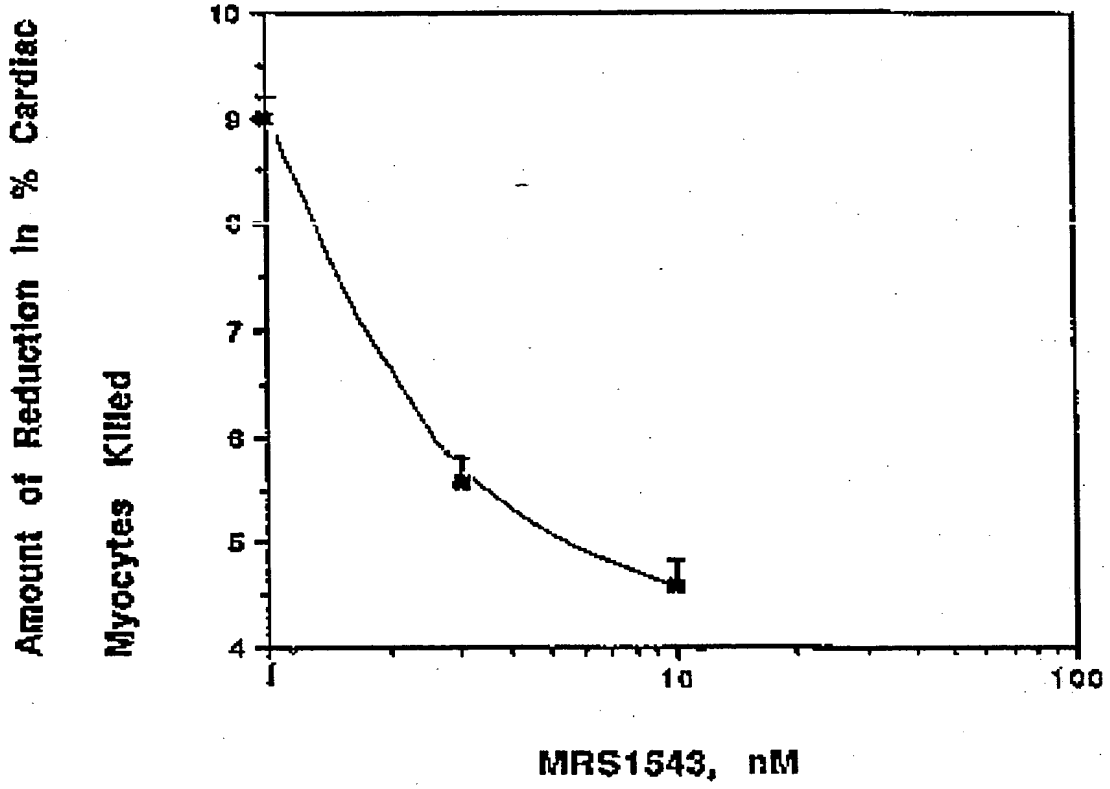

Amount of Reduction in % Cardiac Myocytes Killed
10
9
8
7
6
5
4
1
10
100
MRS1543, nM

Fig. 7A

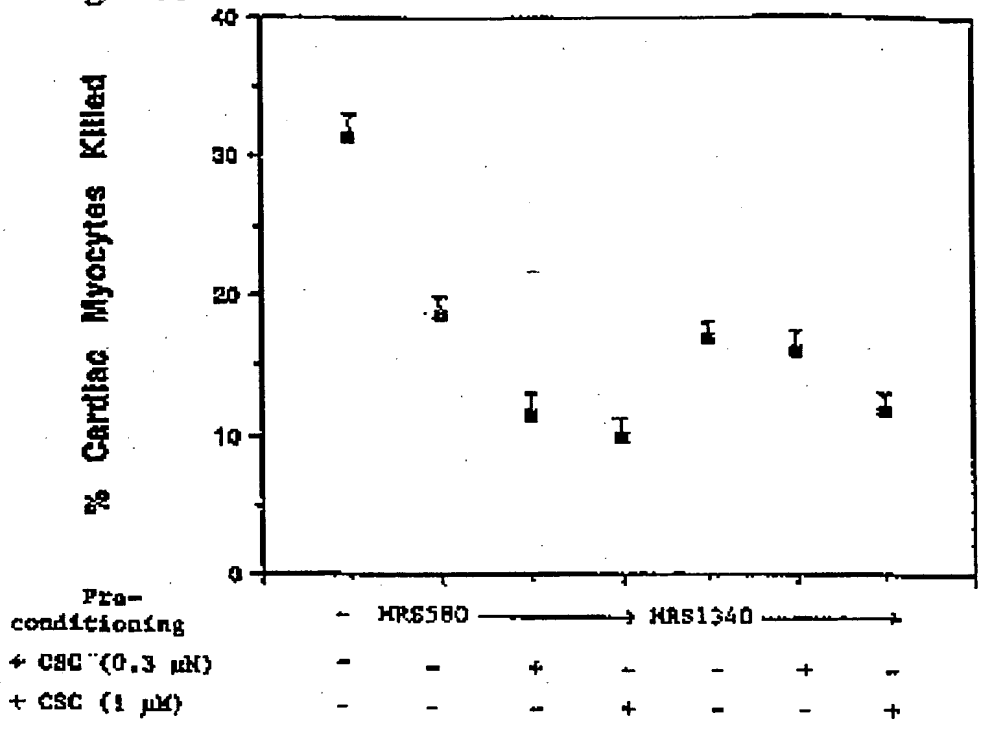

% Cardiac Myocytes Killed
40
30
20
10
0
Pre-conditioning
← HRS580 ——→ HRS1340 ——→
+ CSC (0.3 μM)
+ CSC (1 μM)

Fig. 7B

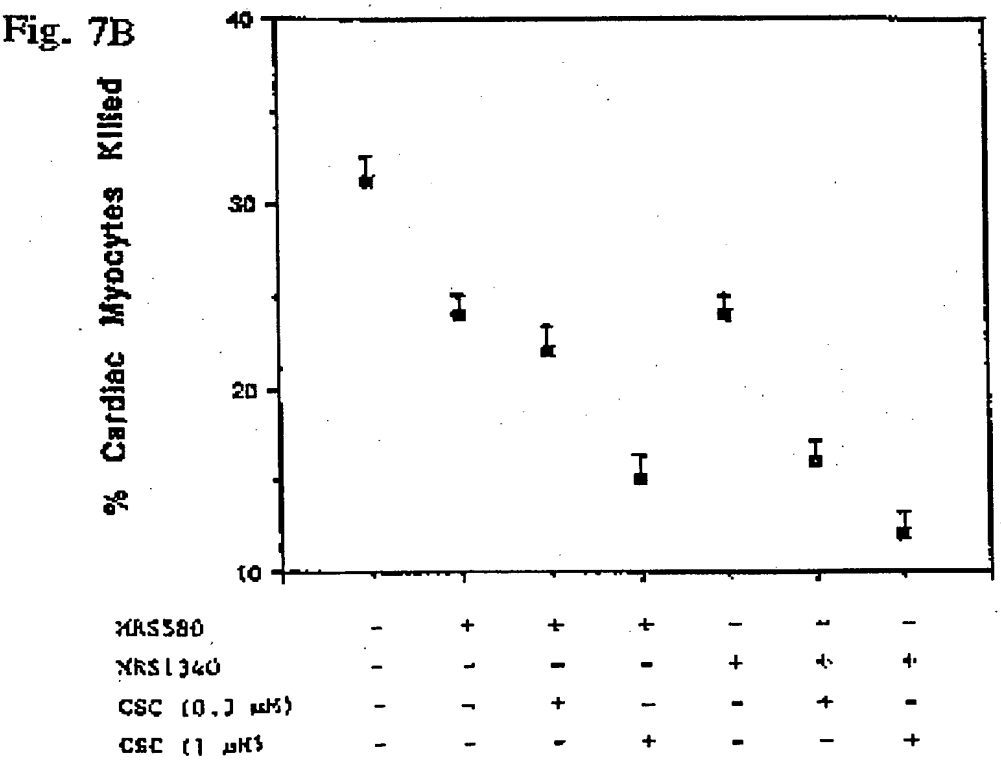

% Cardiac Myocytes Killed
40
30
20
10
HRS580
HRS1340
CSC (0.3 μM)
CSC (1 μM)

Fig. 7C

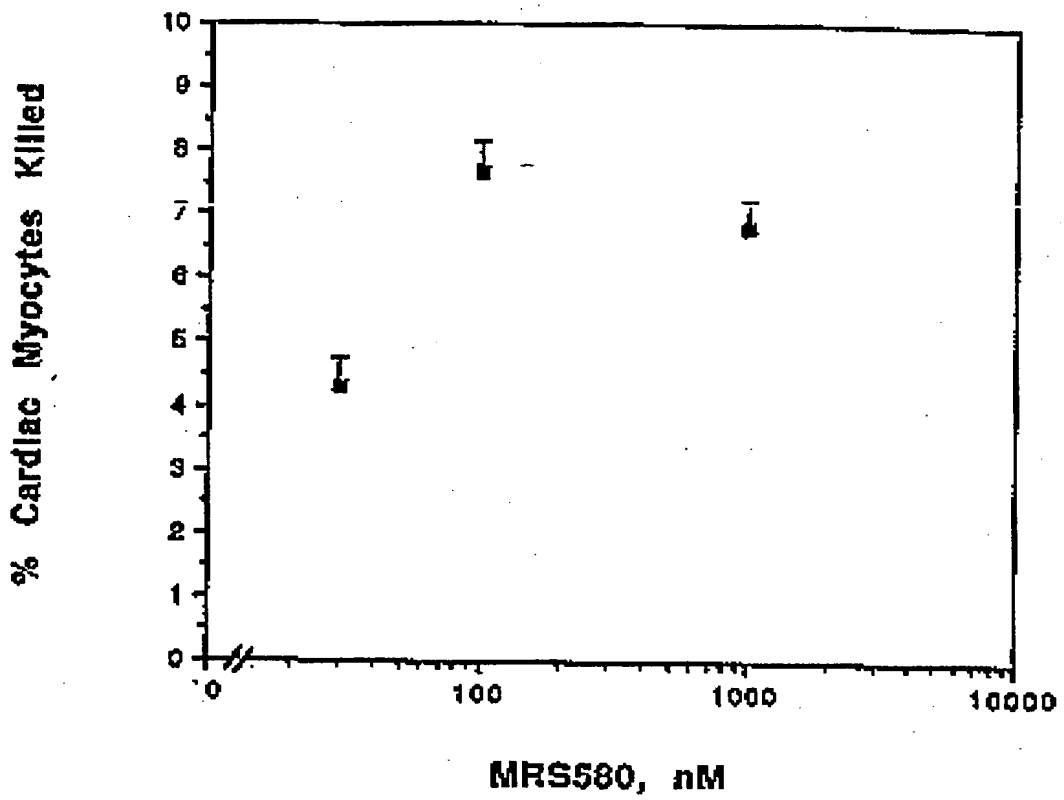

% Cardiac Myocytes Killed
0
1
2
3
4
5
6
7
8
9
10
0
100
1000
10000
MRS580, nM

Fig. 8A

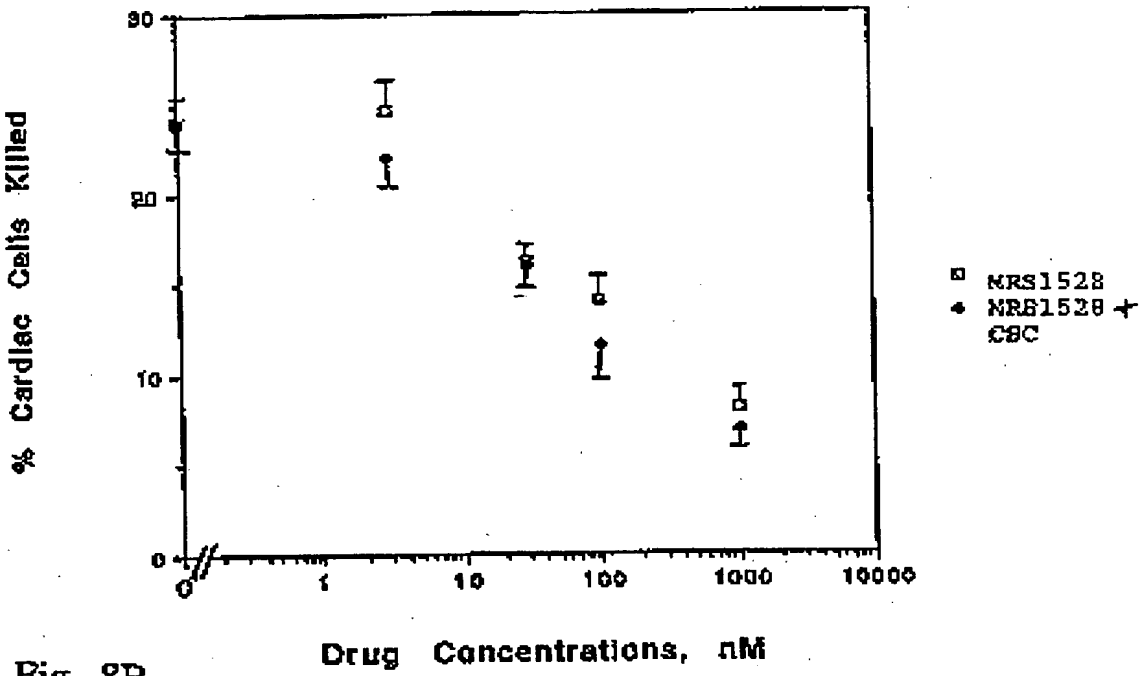
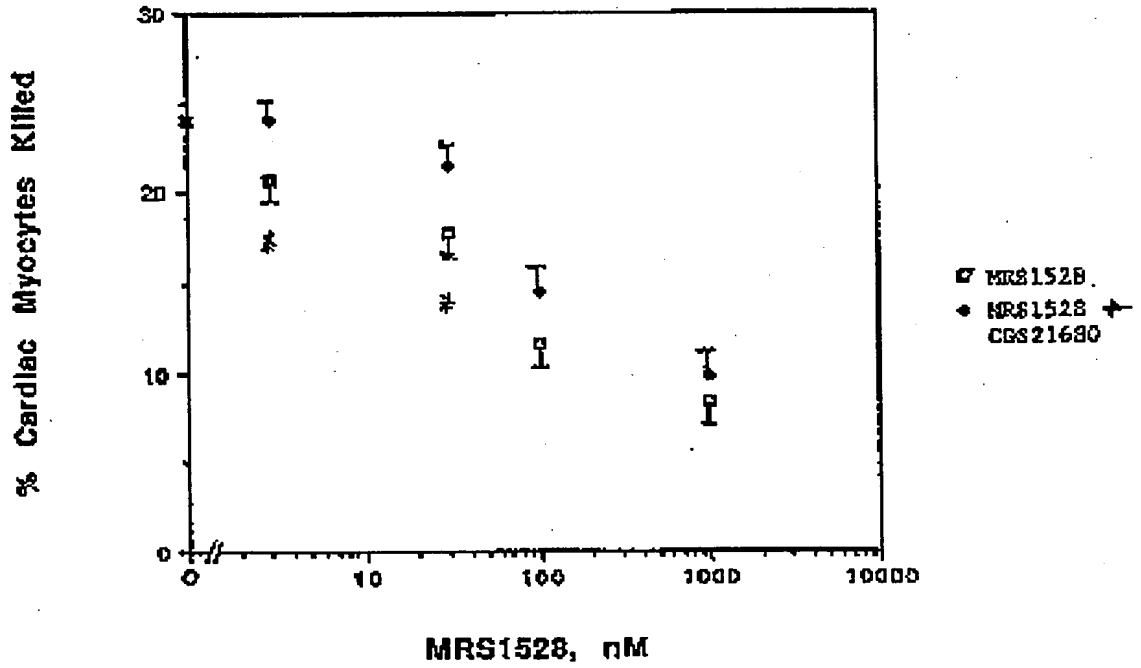


Fig. 8B



* significantly different from those determined in the presence of CGS21680

Fig. 8C

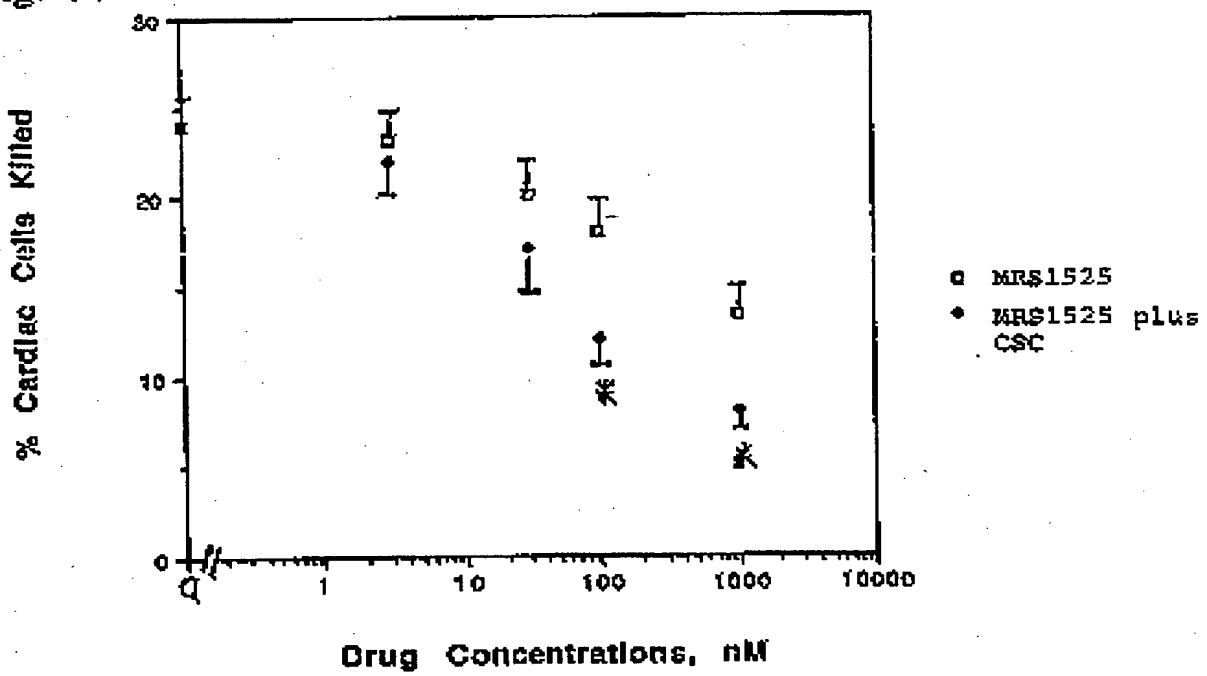

% Cardiac Cells Killed
30
20
10
0
1
10
100
1000
10000
Drug Concentrations, nM
MRS1525
MRS1525 plus CSC

Fig. 8D

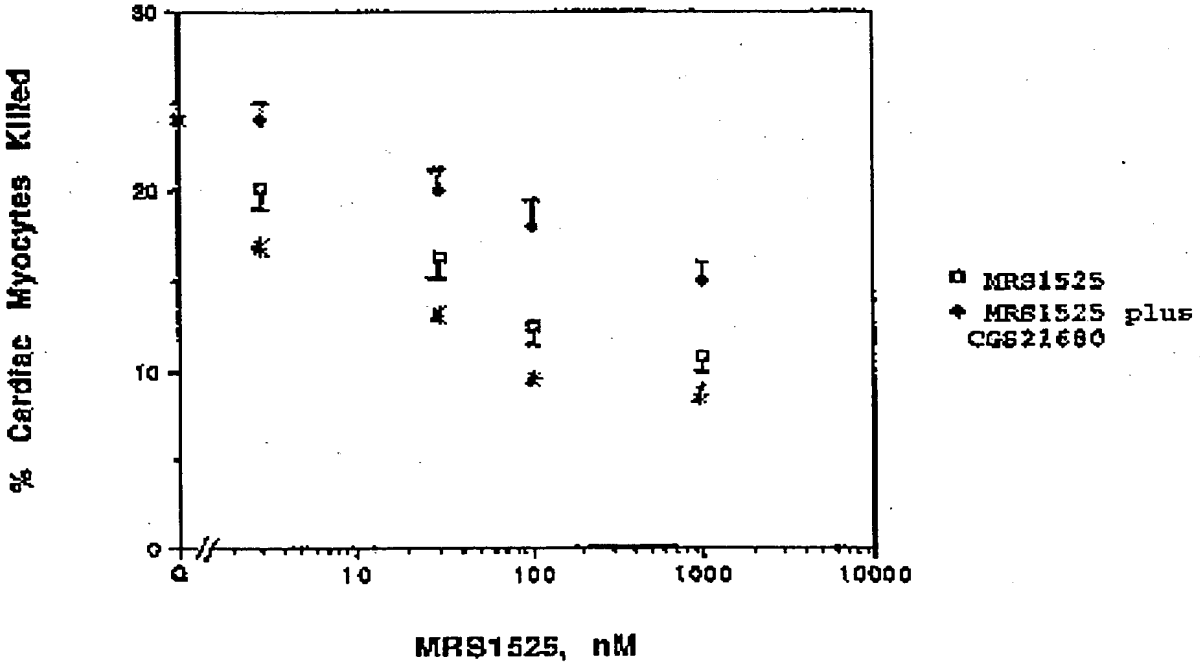
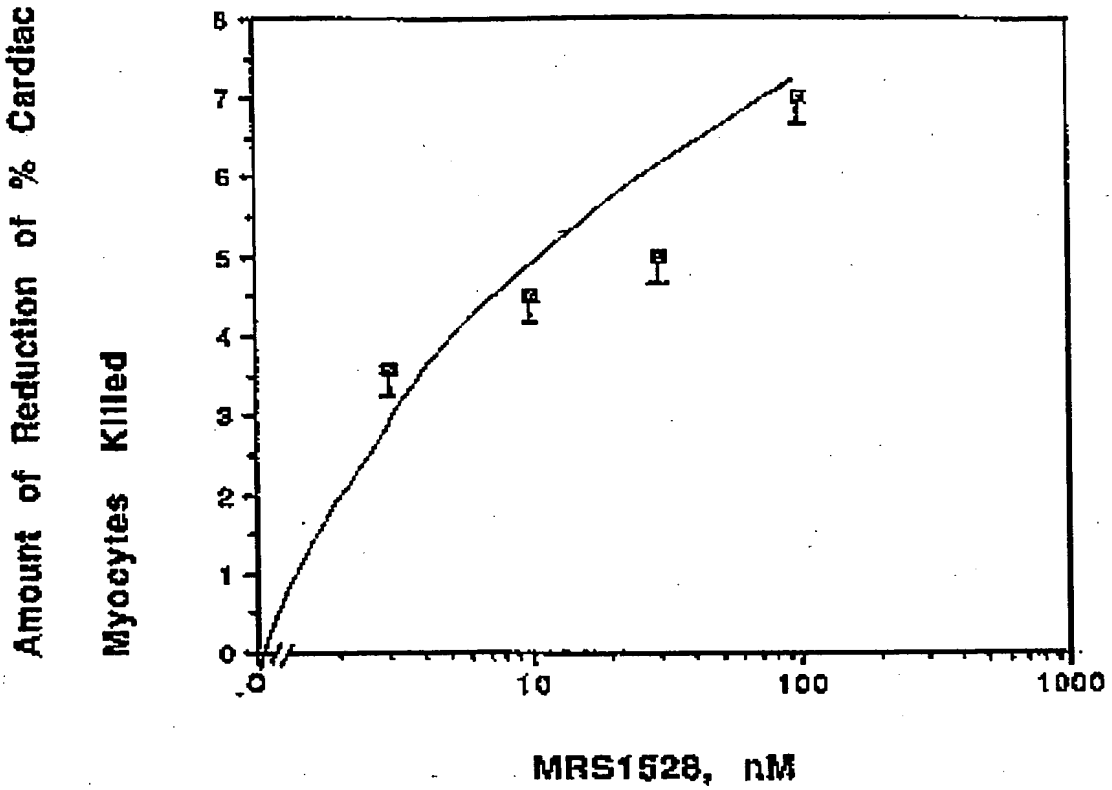

% Cardiac Myocytes Killed
30
20
10
0
10
100
1000
10000
MRS1525, nM
MRS1525
MRS1525 plus CGS21680

Fig. 8E


Amount of Reduction of % Cardiac Myocytes Killed
0
1
2
3
4
5
6
7
8
0
10
100
1000
MRS1528, nM

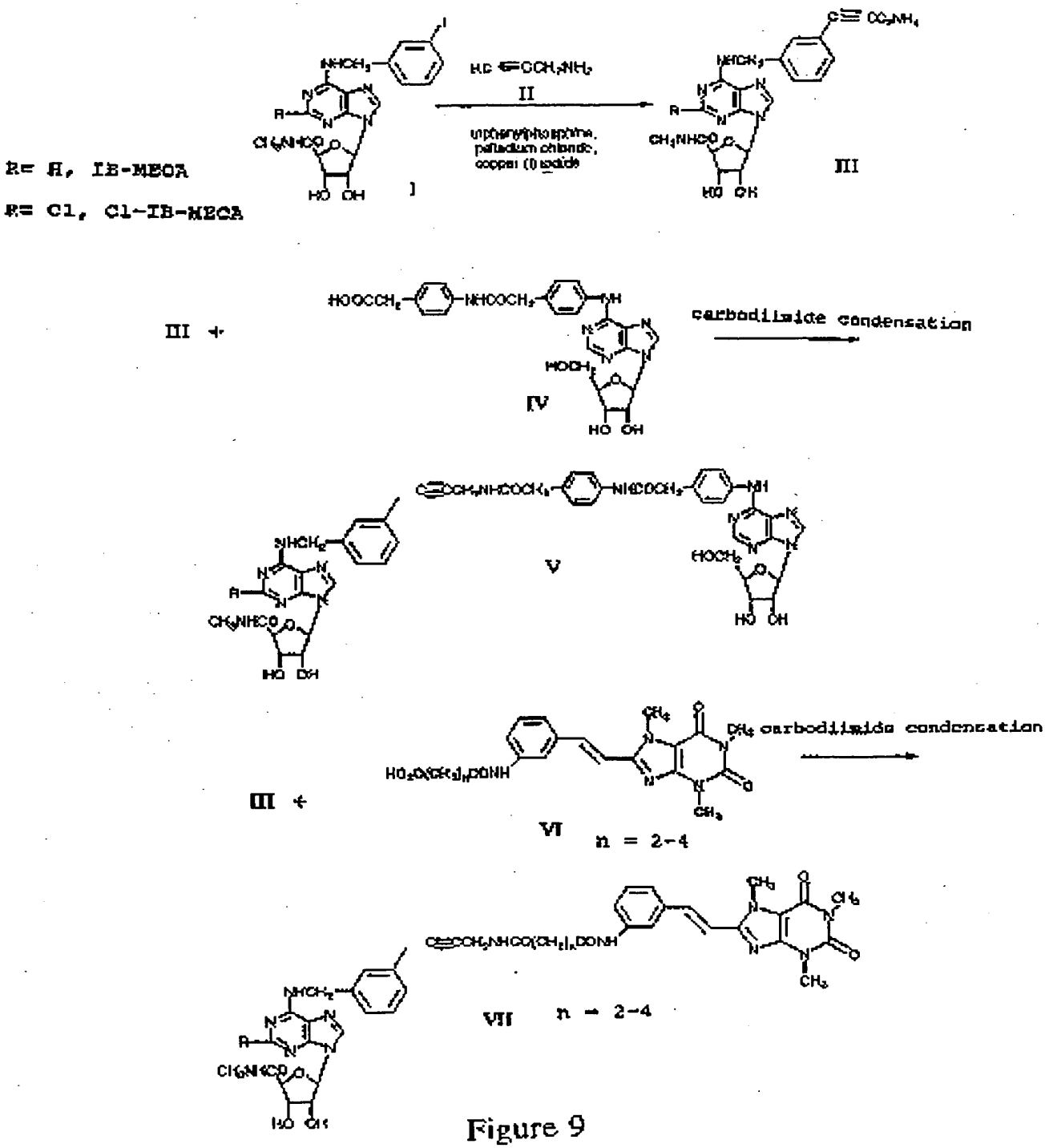
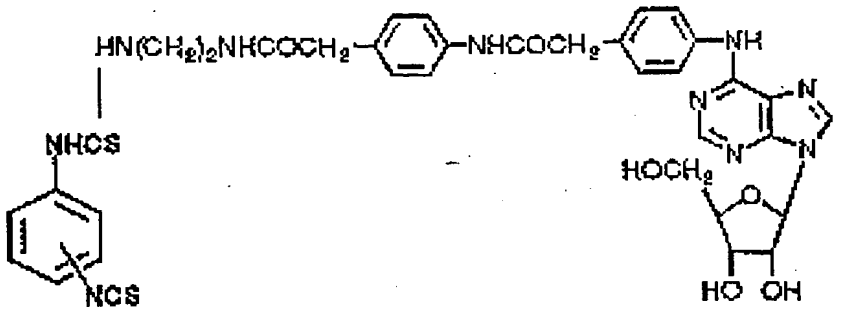

R= H, IB-MECA
R= Cl, Cl-IB-MECA
II
III
carbodiimide condensation
IV
V
VI
n = 2-4
VII
n = 2-4

Figure 9

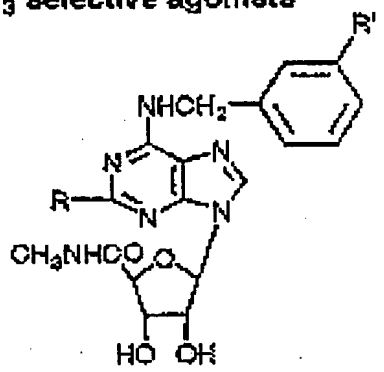
Fig. 10A

Derivatization of A_1 selective agonist for coupling to amine-derivatized A_3 agonist



m- and p-DITC-ADAC

Derivatization of A_3 selective agonists



$R^1 = C{\equiv}CCH_2NHR''$; R = H, Cl

for coupling to isothiocaynates or carboxylic acids:

$R'' = H$, $COCH(R''')NH_2$ [D- or L- amino acid],

for coupling to amines:

$R'' = CSNHC_6H_4NCS$ (p- or m-),

$COCH(R''')NHCSNHC_6H_4NCS$ (p- or m-)

as A_3 agonists for exploring SAR:

$R'' = COCH_3$, $COCH(R''')NHCOOC(CH_3)_3$ [Boc-D- or L- amino acid],

R''' = any naturally occurring amino acid

Fig. 10B

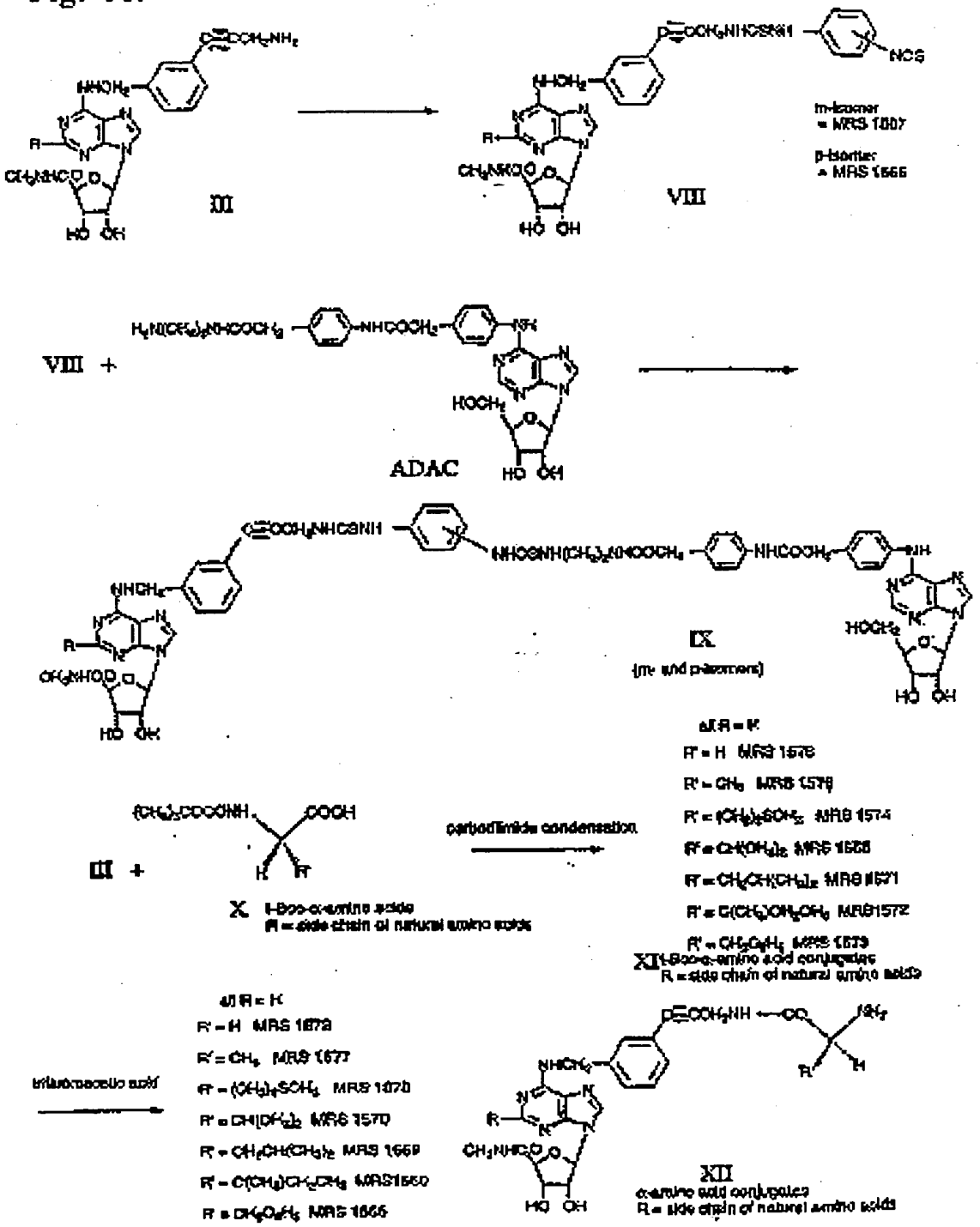
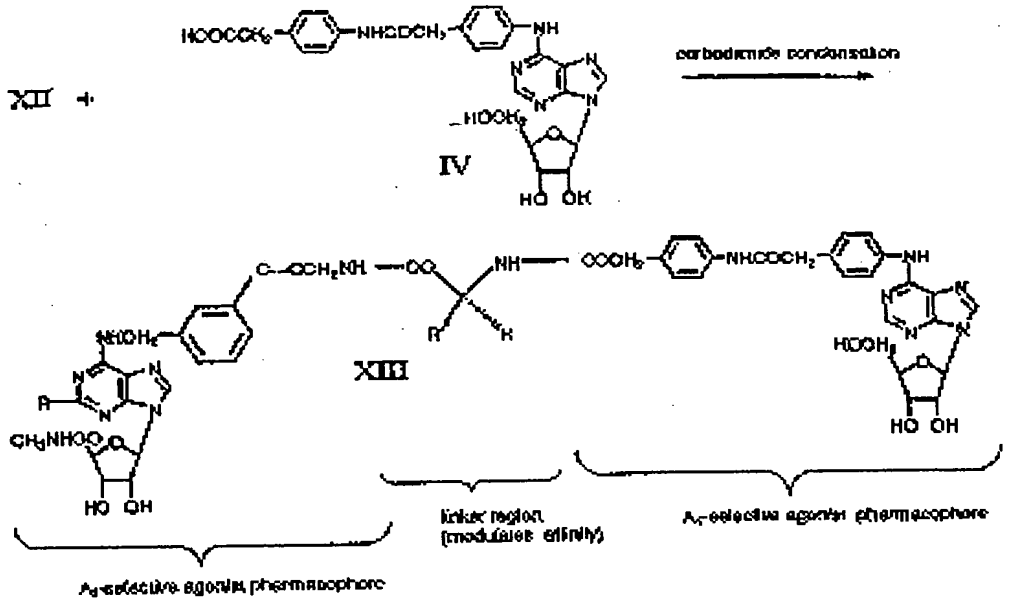

III
VIII
m-isomer = MRS 1667
p-isomer = MRS 1666
VIII +
ADAC
IX
(m- and p-isomers)
a) R = H
R' = H MRS 1576
R' = CH3 MRS 1578
R' = (CH2)4SCH3 MRS 1574
R' = CH(OH)CH3 MRS 1668
R' = CH2CH(CH3)2 MRS 1571
R' = C(CH3)(OH)CH2CH3 MRS1572
R' = CH2C6H5 MRS 1573
III +
X t-Boc-α-amino acids
R = side chain of natural amino acids
carbodiimide condensation
XI t-Boc-α-amino acid conjugates
R = side chain of natural amino acids
trifluoroacetic acid
a) R = H
R' = H MRS 1672
R' = CH3 MRS 1677
R' = (CH2)4SCH3 MRS 1670
R' = CH(OH)CH3 MRS 1570
R' = CH2CH(CH3)2 MRS 1669
R' = C(CH3)(OH)CH2CH3 MRS1560
R' = CH2C6H5 MRS 1665
XII
α-amino acid conjugates
R = side chain of natural amino acids

Fig. 10C

Synthesis of binary conjugate with extended linker



Synthesis of binary conjugate with short linker

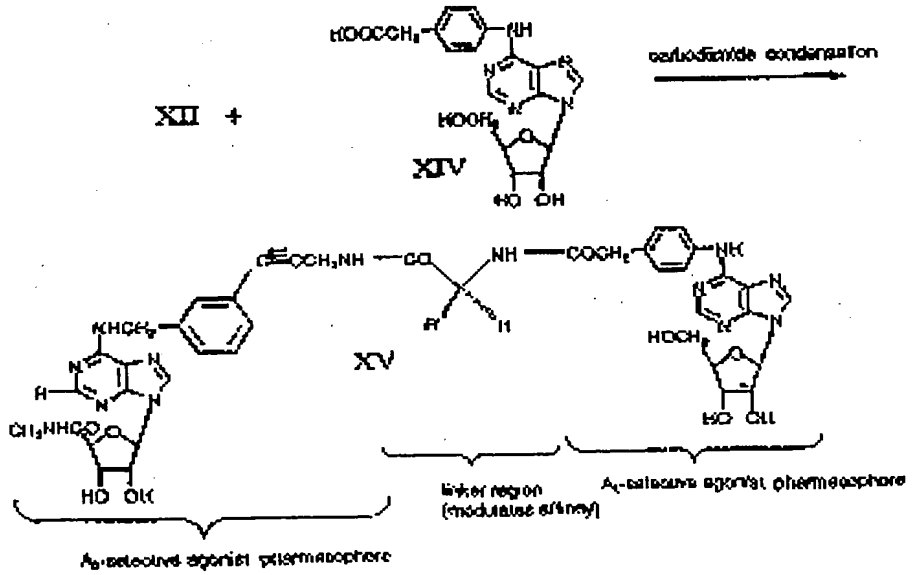


Fig. 11A

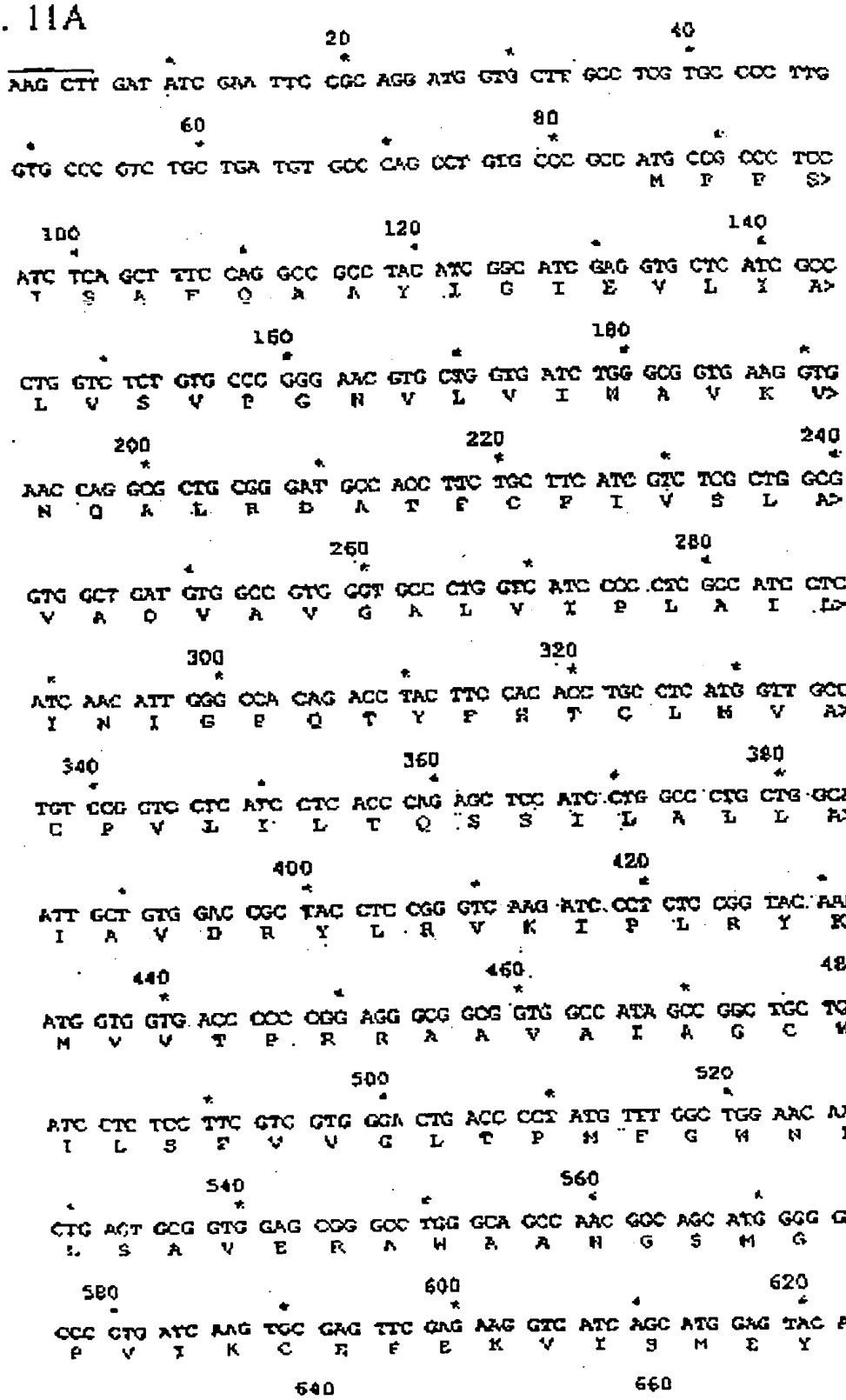

20
40
AAG CTT GAT ATC GAA TTC CGC AGG ATG GTG CTT GCC TCG TGC CCC TTG
60
80
GTG CCC GTC TGC TGA TGT GCC CAG CCT GTG CCC GCC ATG CCG CCC TCC
M P P S>
100
120
140
ATC TCA GCT TTC CAG GCC GCC TAC ATC GGC ATC GAG GTG CTC ATC GCC
T S A F Q A A Y I G I E V L I A>
160
180
CTG GTC TCT GTG CCC GGG AAC GTG CTG GTG ATC TGG GCG GTG AAG GTG
L V S V P G N V L V I W A V K V>
200
220
240
AAC CAG GCG CTG CGG GAT GCC ACC TTC TGC TTC ATC GTC TCG CTG GCG
N Q A L R D A T F C F I V S L A>
260
280
GTG GCT GAT GTG GCC GTC GGT GCC CTG GTC ATC CCC CTC GCC ATC CTC
V A D V A V G A L V I P L A I L>
300
320
ATC AAC ATT GGG CCA CAG ACC TAC TTC CAC ACC TGC CTC ATG GTT GCC
I N I G P Q T Y F H T C L M V A>
340
360
380
TGT CCG GTC CTC ATC CTC ACC CAG AGC TCC ATC CTG GCC CTG CTG GCA
C P V L I L T Q S S I L A L L A>
400
420
ATT GCT GTG GAC CGC TAC CTC CGG GTC AAG ATC CCT CTC CGG TAC AAG
I A V D R Y L R V K I P L R Y K>
440
460
480
ATG GTG GTG ACC CCC CGG AGG GCG GCG GTG GCC ATA GCC GGC TGC TGG
M V V T P R R A A V A I A G C W>
500
520
ATC CTC TCC TTC GTC GTG GGA CTG ACC CCT ATG TTT GGC TGG AAC AAT
I L S F V V G L T P M F G W N N>
540
560
CTG AGT GCG GTG GAG CGG GCC TGG GCA GCC AAC GGC AGC ATG GGG GAG
L S A V E R A W A A N G S M G E>
580
600
620
CCC CTG ATC AAG TGC GAG TTC GAG AAG GTC ATC AGC ATG GAG TAC ATG
P V I K C E F E K V I S M E Y M>
640
660

Fig. 11B

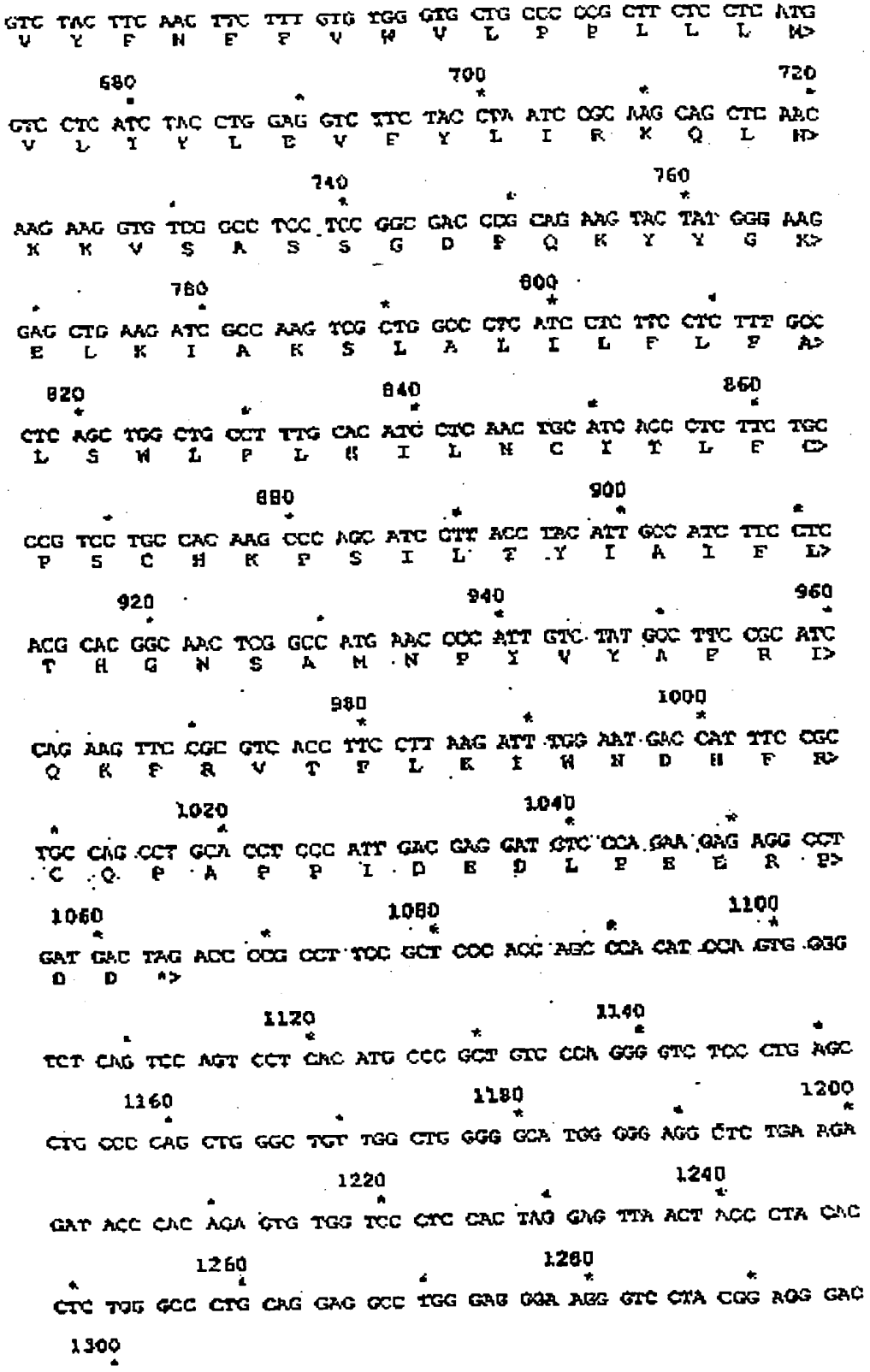


Fig. 12A

```
                10                  20                  30                  40
·CAA TTT TCA GCT GTT CCT TGC TCA ATA ATA ACT TTT TTA TCA CCA AGA

 50                  60                  70                  80                  90
TAT CTC TCT AAG TTT TTG ACA TAT TCC TCA TTT GTT TTC ATA AAA GTT

  100                 110                 120                 130                 140
TTC TTA TTT TCT TAG AAA AAT AAG TTA CTA AAA GTC ATA TAT CAT TGT

      150                 160                 170                 180                 190
ATA TCT TCA AAA TAT TGC TTA AAA CTA GGA CTT GTA TTT AAA TGT TTT

        200                 210                 220                 230                 240
TTC TTC TTA AAG ACA ATT TGC AGG TGC CCT CAC GAA CCC TGA AGC TGG

            250                 260                 270                 280
GCT GAG CCA TGA TGC TGC TGC CAG AAC CCC TGC AGA GGG CCT GGT TTC

290                 300                 310                 320                 330
AGG AGA CTC AGA GTC CTC TGT GAA AAA GCC CTT GGA GAG CGC CCC AGC

   340                 350                 360                 370                 380
AGG GCT GCA CTT GGC TCC TGT GAG GAA GGG GCT CAG GGG TCT GGG CCC

      390                 400                 410                 420                 430
CTC CGC CTG GGC CGG GCT GGG AGC CAG GCG GGC GGC TGC GCT GCA GCA

          440                 450                 460                 470                 480
AAT GGA CCG TGA GCT GGC CCA GCC CGC GTC CGT GCT GAG CCT GCC TGT

             490                 500                 510                 520                 530
CGT CTG TGG CC ATG CCC ATC ATG GGC TCC TCG GTG TAC ATC ACG GTG GAG
                M   P   I   M   G   S   S   V   Y   I   T   V   E>

             540                 550                 560                 570
CTG GCC ATT GCT GTG CTG GCC ATC CTG GGC AAT GTG CTG GTG TGC TGG
  L   A   I   A   V   L   A   I   L   G   N   V   L   V   C   W>

580                 590                 600                 610                 620
GCC GTG TGG CTC AAC AGC AAC CTG CAG AAC GTC ACC AAC TAC TTT GTG
  A   V   W   L   N   S   N   L   Q   N   V   T   N   Y   F   V>

    630                 640                 650                 660                 670
GTG TCA CTC GCG GCG GCC GAC ATC GCA GTG GGT GTG CTC GCC ATC CCC
  V   S   L   A   A   A   D   I   A   V   G   V   L   A   I   P>

        680                 690                 700                 710                 720
TTT GCC ATC ACC ATG AGC ACC GGG TTC TGC GCT GCC TGC CAC GGC TGC
F   A   I   T   M   S   T   G   F   C   A   A   C   H   G   C
```

Fig. 12B

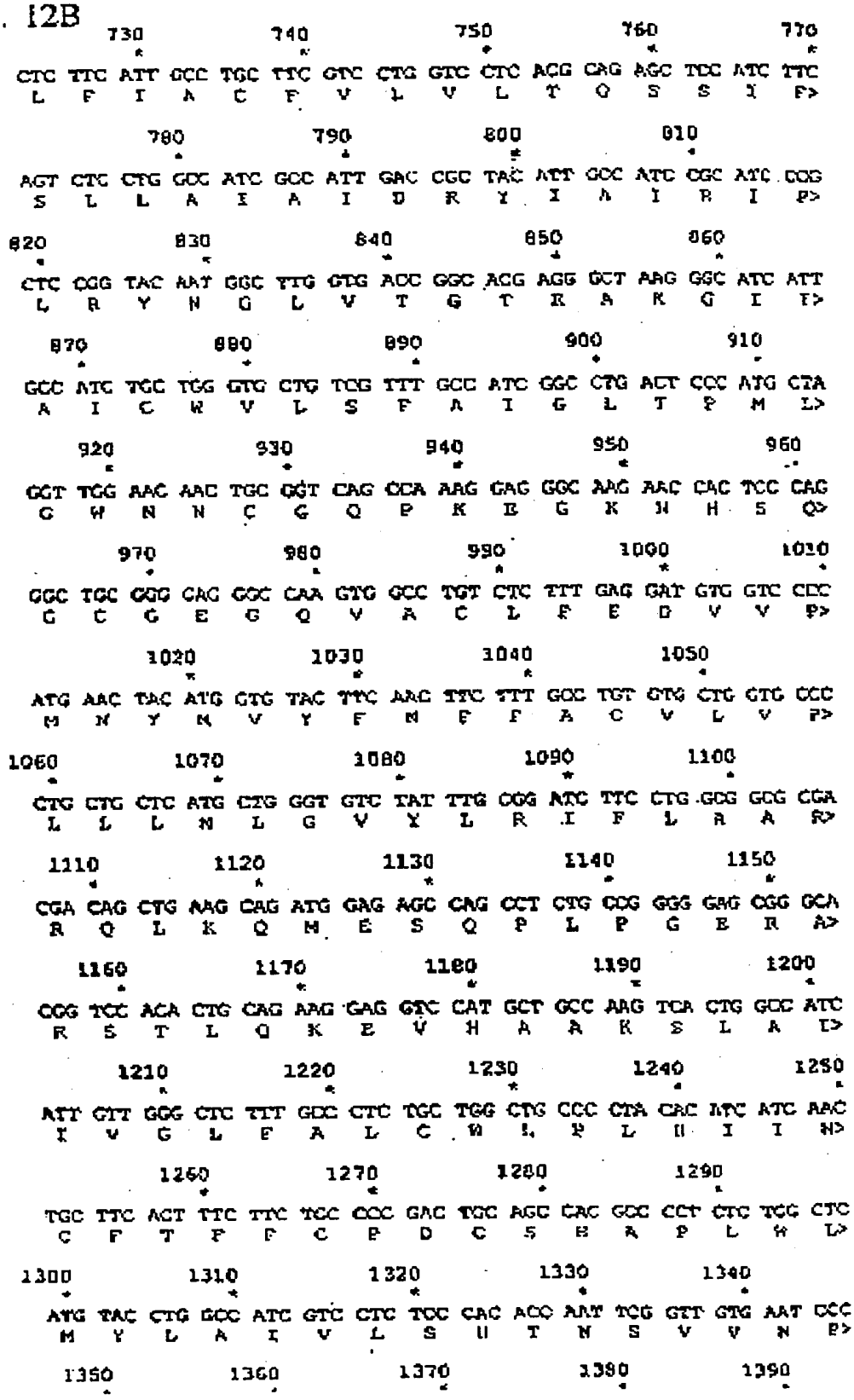
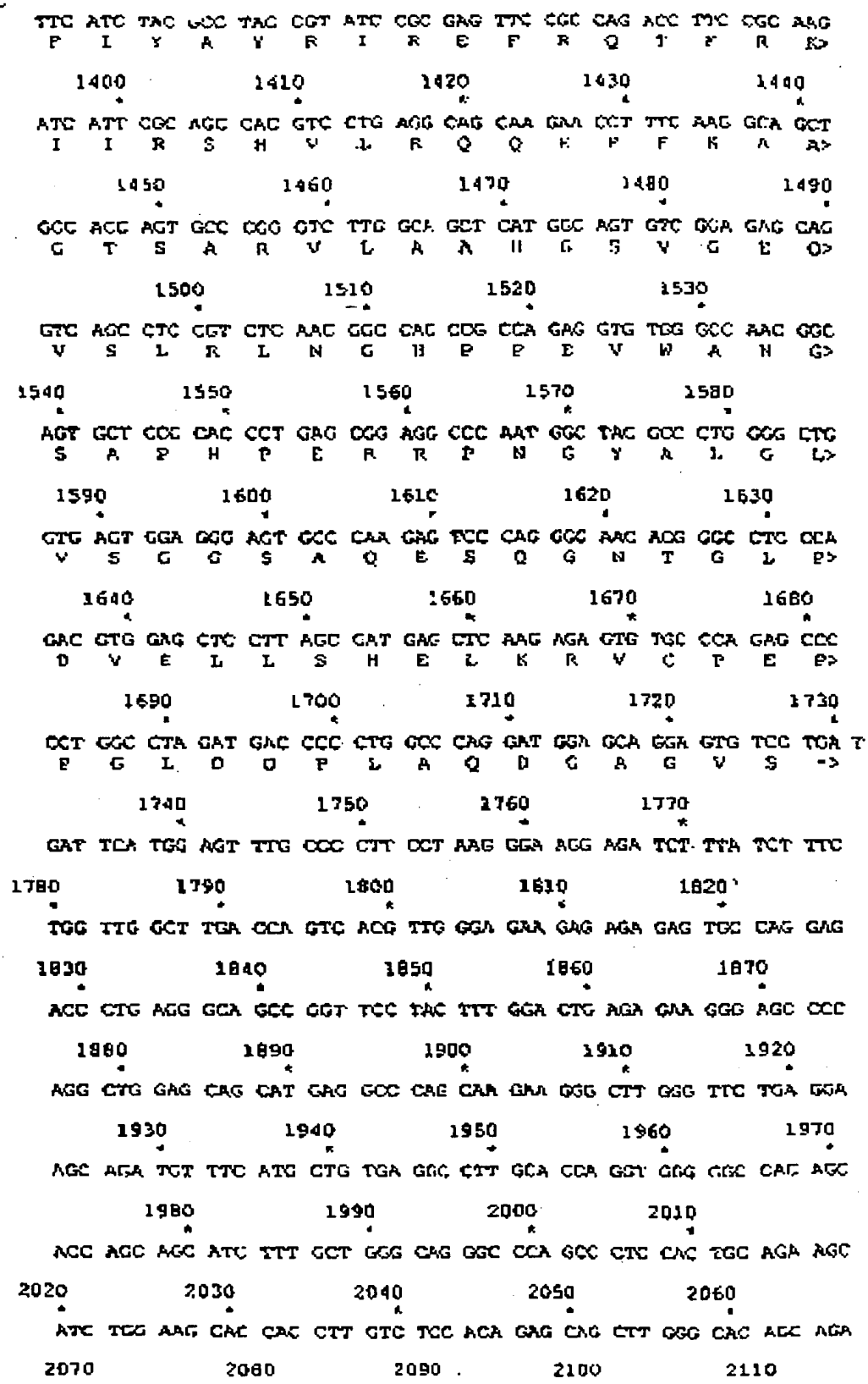


Fig. 12C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P I Y A Y R I R E F R Q T F R K>
1400 1410 1420 1430 1440
ATC ATT CGC AGC CAC GTC CTG AGG CAG CAA GAA CCT TTC AAG GCA GCT
I I R S H V L R Q Q E P F K A A>
1450 1460 1470 1480 1490
GCC ACG AGT GCC CGG GTC TTG GCA GCT CAT GGC AGT GTC GGA GAG CAG
G T S A R V L A A H G S V G E Q>
1500 1510 1520 1530
GTC AGC CTC CGT CTC AAC GGC CAC CCG CCA GAG GTG TGG GCC AAC GGC
V S L R L N G H P P E V W A N G>
1540 1550 1560 1570 1580
AGT GCT CCC CAC CCT GAG CGG AGG CCC AAT GGC TAC GCC CTG GGG CTG
S A P H P E R R P N G Y A L G L>
1590 1600 1610 1620 1630
GTG AGT GGA GGG AGT GCC CAA GAG TCC CAG GGC AAC ACG GCC CTC CCA
V S G G S A Q E S Q G N T G L P>
1640 1650 1660 1670 1680
GAC GTG GAG CTC CTT AGC CAT GAG CTC AAG AGA GTG TGC CCA GAG CCC
D V E L L S H E L K R V C P E P>
1690 1700 1710 1720 1730
CCT GGC CTA GAT GAC CCC CTG GCC CAG GAT GGA GCA GGA GTG TCC TGA T
P G L D D P L A Q D G A G V S ->
1740 1750 1760 1770
GAT TCA TGG AGT TTG CCC CTT CCT AAG GGA AGG AGA TCT TTA TCT TTC
1780 1790 1800 1810 1820
TGG TTG GCT TGA CCA GTC ACG TTG GGA GAA GAG AGA GAG TGC CAG GAG
1830 1840 1850 1860 1870
ACC CTG AGG GCA GCC GGT TCC TAC TTT GGA CTG AGA GAA GGG AGC CCC
1880 1890 1900 1910 1920
AGG CTG GAG CAG CAT GAG GCC CAG CAA GAA GGG CTT GGG TTC TGA GGA
1930 1940 1950 1960 1970
AGC AGA TGT TTC ATG CTG TGA GGC CTT GCA CCA GGT GGG GGC CAC AGC
1980 1990 2000 2010
ACC AGC AGC ATC TTT GCT GGG CAG GGC CCA GCC CTC CAC TGC AGA AGC
2020 2030 2040 2050 2060
ATC TGG AAG CAC CAC CTT GTC TCC ACA GAG CAG CTT GGG CAC AGC AGA
2070 2080 2090 2100 2110

Fig. 12D

```
                                                                    *
CTG GCC TGG CCC TGA GAC TGG GGA GTG GCT CCA ACA GCC TCC TGC CAC

  2120          2130          2140          2150          2160
CCA CAC ACC ACT CTC CCT AGA CTC TCC TAG GGT TCA GGA GCT GCT GGG

      2170          2180          2190          2200          2210
CCC AGA GGT GAC ATT TGA CTT TTT TTC CAG GAA AAA TGT AAG TGT GAG

            2220          2230          2240          2250
GAA ACC CTT TTT ATT TTA TTA CCT TTC ACT CTC TGG CTG CTG GGT CTG

2260          2270          2280          2290          2300
CCG TCG GTC CTG CTG CTA ACC TGG CAC CAG AGC CTC TGC CCG GGG AGC

  2310          2320          2330          2340          2350
CTC AGG CAG TCC TCT CCT GCT GTC ACA GCT GCC ATC CAC TTC TCA GTC

    2360          2370          2380          2390          2400
CCA GGG CCA TCT CTT GGA GTG ACA AAG CTG GGA TCA AGG ACA GGG AGT

      2410          2420          2430          2440          2450
TGT AAC AGA GCA GTG CCA GAG CAT GGG CCC AGG TCC CAG GGG AGA GGT

            2460          2470          2480          2490
TGG GGC TGG CAG GCC ACT GGC ATG TGC TGA GTA GCG CAG AGC TAC CCA

2500          2510          2520          2530          2540
GTG AGA GGC CTT GTC TAA CTG CCT TTC CTT CTA AAG GGA ATG TTT TTT

  2550          2560          2570
TCT GAG ATA AAA TAA AAA CGA GCC ACA G
```

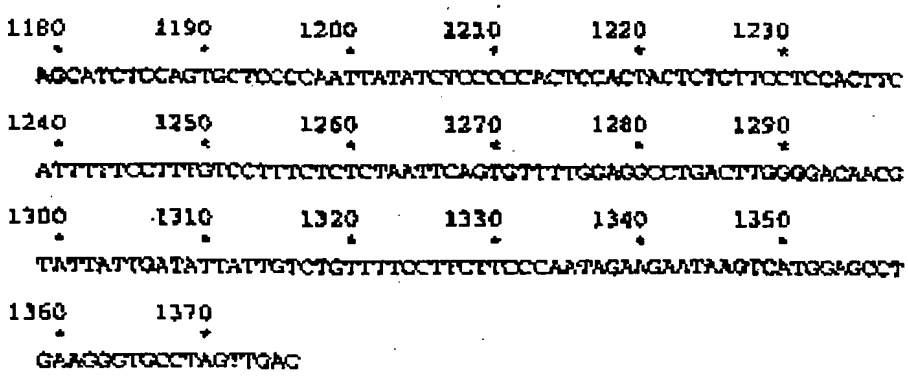
Fig. 13A

```
         10         20         30         40         50         60
         *          *          *          *          *          *
-GGACCTCTGGCAAGACGTCTGCCGACAGCTAGGCCCACTGGCCCTACAGACGGATCTTGC

         70         80         90        100        110
         *          *          *          *          *
TGGCTCAGCTGTCCCTGTGGAGGTTCCCCTGGGAACGCAAG ATG CCC AAC AAC
                                          Met Pro Asn Asn>

      120        130        140        150
      *          *          *          *
AGC ACT GCT CTG TCA TTG GCC AAT GTT ACC TAC ATC ACC ATG GAA
Ser Thr Ala Leu Ser Leu Ala Asn Val Thr Tyr Ile Thr Met Glu>

160        170        180        190        200
*          *          *          *          *
ATT TTC ATT GGA CTC TGC GCC ATA GTG GGC AAC GTG CTG GTC ATC
Ile Phe Ile Gly Leu Cys Ala Ile Val Gly Asn Val Leu Val Ile>

      210        220        230        240
      *          *          *          *
TGC GTG GTC AAG CTG AAC CCC AGC CTG CAG ACC ACC ACC TTC TAT
Cys Val Val Lys Leu Asn Pro Ser Leu Gln Thr Thr Thr Phe Tyr>

250        260        270        280        290
*          *          *          *          *
TTC ATT GTC TCT CTA GCC CTG GCT GAC ATT GCT GTT GGG GTG CTG
Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala Val Gly Val Leu>

      300        310        320        330
      *          *          *          *
GTC ATG CCT TTG GCC ATT GTT GTC AGC CTG GGC ATC ACA ATC CAC
Val Met Pro Leu Ala Ile Val Val Ser Leu Gly Ile Thr Ile His>

340        350        360        370        380
*          *          *          *          *
TTC TAC AGC TGC CTT TTT ATG ACT TGC CTA CTG CTT ATC TTT ACC
Phe Tyr Ser Cys Leu Phe Met Thr Cys Leu Leu Leu Ile Phe Thr>

      390        400        410        420
      *          *          *          *
CAC GCC TCC ATC ATG TCC TTG CTG GCC ATC GCT GTG GAC CGA TAC
His Ala Ser Ile Met Ser Leu Leu Ala Ile Ala Val Asp Arg Tyr>

430        440        450        460        470
*          *          *          *          *
TTG CGG GTC AAG CTT ACC GTC AGA TAC AAG AGG GTC ACC ACT CAC
Leu Arg Val Lys Leu Thr Val Arg Tyr Lys Arg Val Thr Thr His>

      480        490        500        510
      *          *          *          *
AGA AGA ATA TGG CTG GCC CTG GGC CTT TGC TGG CTG GTG TCA TTC
Arg Arg Ile Trp Leu Ala Leu Gly Leu Cys Trp Leu Val Ser Phe>

520        530        540        550        560
*          *          *          *          *
CTG GTG GGA TTG ACC CCC ATG TTT GGC TGG AAC ATG AAA CTG ACC
Leu Val Gly Leu Thr Pro Met Phe Gly Trp Asn Met Lys Leu Thr>

      570        580        590        600
```

Fig. 13B

```
TCA GAG TAC CAC AGA AAT GTC ACC TTC CTT TCA TGC CAA TTT GTT
Ser Glu Tyr His Arg Asn Val Thr Phe Leu Ser Cys Gln Phe Val>

610         620         630         640         650
TCC GTC ATG AGA ATG GAC TAC ATG GTA TAC TTC AGC TTC CTC ACC
Ser Val Met Arg Met Asp Tyr Met Val Tyr Phe Ser Phe Leu Thr>

      660         670         680         690
TGG ATT TTC ATC CCC CTG GTT GTC ATG TGC GCC ATC TAT CTT GAC
Trp Ile Phe Ile Pro Leu Val Val Met Cys Ala Ile Tyr Leu Asp>

700         710         720         730         740
ATC TTT TAC ATC ATT CGG AAC AAA CTC AGT CTG AAC TTA TCT AAC
Ile Phe Tyr Ile Ile Arg Asn Lys Leu Ser Leu Asn Leu Ser Asn>

      750         760         770         780
TCC AAA GAG ACA GGT GCA TTT TAT GGA CGG GAG TTC AAG ACG GCT
Ser Lys Glu Thr Gly Ala Phe Tyr Gly Arg Glu Phe Lys Thr Ala>

790         800         810         820         830
AAG TCC TTG TTT CTG GTT CTT TTC TTG TTT GCT CTG TCA TGG CTG
Lys Ser Leu Phe Leu Val Leu Phe Leu Phe Ala Leu Ser Trp Leu>

      840         850         860         870
CCT TTA TCT ATC ATC AAC TGC ATC ATC TAC TTT AAT GGT GAG GTA
Pro Leu Ser Ile Ile Asn Cys Ile Ile Tyr Phe Asn Gly Glu Val>

880         890         900         910         920
CCA CAG CTT GTG CTG TAC ATG GGC ATC CTG CTG TCC CAT GCC AAC
Pro Gln Leu Val Leu Tyr Met Gly Ile Leu Leu Ser His Ala Asn>

      930         940         950         960
TCC ATG ATG AAC CCT ATC GTC TAT GCC TAT AAA ATA AAG AAG TTC
Ser Met Met Asn Pro Ile Val Tyr Ala Tyr Lys Ile Lys Lys Phe>

970         980         990         1000        1010
AAG GAA ACC TAC CTT TTG ATC CTC AAA GCC TGT GTG GTC TGC CAT
Lys Glu Thr Tyr Leu Leu Ile Leu Lys Ala Cys Val Val Cys His>

      1020        1030        1040        1050
CCC TCT GAT TCT TTG GAC ACA AGC ATT GAG AAG AAT TCT GAG TAG
Pro Ser Asp Ser Leu Asp Thr Ser Ile Glu Lys Asn Ser Glu ***>

1060        1070        1080        1090        1100        1110
TTATCCATCAGAGATGACTCTGTCTCAGTGACCTTCAGATTCCCCATCAACAAACACTTG

1120        1130        1140        1150        1160        1170
AGCGCCTGTATGCCTGGGCCAAGCGATTTTTACATCCTTGATTACTTCCACTCAGGTGCG
```

Fig. 13 C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1240 1250 1260 1270 1280 1290
ATTTTTCCTTTGTCCTTTCTCTCTAATTCAGTGTTTTGGAGGCCTGACTTGGGGACAACG
1300 1310 1320 1330 1340 1350
TATTATTCATATTATTGTCTGTTTTCCTTCTTCCCAATAGAAGAATAAGTCATGGAGCCT
1360 1370
GAAGGGTGCCTAGTTGAC